The impact on receiving waters of pharmaceutical residues and antibiotic resistant faecal bacteria found in urban waste water effluents

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Abstract

Pharmaceuticals intended for human use are frequently detected in the aquatic environment. This is predominantly from their excretion following ingestion and subsequent discharge in domestic sewage. Wastewater treatment provides an opportunity to control their release to surface waters however, their removal is often incomplete. This thesis addresses this pharmaceutical pathway and the potential impact on the aquatic environment.

The progress of bezafibrate, carbamazepine, ciprofloxacin and clarithromycin were monitored through the treatment stages (screened sewage, settled sewage and final effluent) of a large urban wastewater treatment plant (WWTP) and in surface waters up-stream and down-stream of the effluent discharge point. All except clarithromycin were detected in the screened sewage (369 – 2696 ng/L). Reductions in the pharmaceutical concentrations throughout the WWTP (22.5 – 94.3 %) indicate the removal of these compounds is variable. Bezafibrate and carbamazepine were observed at higher concentrations (67.5 - 305.5 ng/L) in surface water down-stream of the effluent discharge point compared to up-stream (31.0 – 116.7 ng/L).

The presence of antibiotics in the environment may contribute to the dissemination of antibiotic resistance. The second part of this thesis monitors the prevalence of resistant faecal bacteria through WWTPs and in surface waters. Determination of antibiotic minimum inhibitory concentration (MIC) values for *E.coli* and *E.faecium* indicated that the WWTP did not influence the proportions of the resistant bacterial species. Elevated levels of *E.coli* with acquired ciprofloxacin resistance increased from not detectable in surface waters up-stream

to 9.3% down-stream of the WWTP discharge point. The need for standardisation of the interpretation of MIC data is addressed.

The potential of ciprofloxacin within surface water to select for ciprofloxacin resistant *E.coli* were investigated through microcosm studies in the third part of this study. A significant increase ($p < 0.05$) in the level of resistant *E.coli* was observed in microcosms exposed to ≥ 5 μg/L ciprofloxacin. At the ciprofloxacin levels typically detected in surface waters receiving treated effluent (<300 ng/L), the levels of resistance amongst *E.coli* were maintained.

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Abbreviations

CBP	clinical breakpoint
ECOff	epidemiological cut off value
LC-MS	liquid chromatography mass spectrometry
LOD	limit of detection
MIC	minimum inhibitory concentration
PPCP	pharmaceuticals and personal care products
TBX	tryptone bile x glucuronide
TSS	total suspended solids
SIM	selective ion monitoring
WWTP	wastewater treatment plant

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1 Introduction

Water is essential to life and therefore it is imperative to protect and maintain water quality. However, the quality of natural waters is under threat from the chemical substances discharged in industrial and domestic waste. Consequently, water quality is enforced under legislation such as, the Water Framework Directive (Defra, 2009) in Europe and the Clean Water Act (EPA, 2010) in the USA. Currently, legislation is focused on reducing 'priority pollutants' that include persistent organic compounds (e.g. polyaromatic hydrocarbons and dioxins) and heavy metals. However, a new class of emerging pollutants which is gaining increasing attention includes pharmaceutical compounds and the active ingredients used in personal care products (collectively termed PPCPs). With improving analytical techniques, these active compounds, although at trace concentrations (ng/L to µg/L) have been detected in wastewaters (Gracia-Lor et al., 2010; Gros et al., 2010; Golet et al., 2001) and natural waters (Kasprzyk-Hordern et al., 2008a; Kasprzyk-Hordern et al., 2007; Cha et al., 2006; Roberts et al., 2006; Farré et al., 2001) although their environmental fate and the possible risks that they pose to aquatic ecosystems still require further elucidation (Boxall, 2004).

Depending on their pharmokinetic properties, PPCPs will be excreted after ingestion, either as the parent compound or as metabolites and discharged into domestic wastewaters (Garcia-Ac et al., 2009). Due to incomplete elimination during wastewater treatment or as a result of their presence in agricultural run off after the application of manure to fields as a fertilizer, PPCPs are released into receiving waters (Carballa et al., 2004; Boxall et al., 2002). The removal rate of PPCPs from wastewaters varies according to the physico-chemical and

biological properties of the active compound, and is affected by several factors, such as the treatment process employed, activated sludge (type and age), environmental temperature, and light exposure (Farré et al., 2007). PPCPs may be released from wastewater treatment processes in a modified form, as either hydroxylated or conjugated transformation products (Quintana et al., 2005). In the aquatic environment, PPCPs are subjected to multiple environmental factors, either biotic (e.g. bacterial or fungal) or abiotic (e.g. sorption, photolysis, oxidation, hydrolysis and reduction) processes (Kümmerer, 2009). In contrast to persistent organic pollutants such as organochlorine compounds (e.g. dichlorodiphenyltrichloroethane), that were used as insecticides and pesticides, PPCPs are more vulnerable to degradation. The continual release of PPCPs into the environment leads to their classification as pseudo persistent pollutants (Stackelberg et al., 2004; Ternes, 1998).

Antibiotics are a successful family of pharmaceuticals used in medicine to prevent and treat infections caused by micro-organisms such as bacteria and fungi. Their importance in medicine in the fight against infectious diseases accounts for their large-scale usage which in turn is associated with the emergence of micro-organisms resistant to the antibiotics used against them (Henriques et al., 2006). Bacterial resistance to antibiotics increases the difficulty in treating both hospital and community acquired infections and this is therefore of great concern to public health. Consequently, there has been extensive research in the clinical domain into the development of antibiotic resistance. In recent years, there has been an increasing interest in the occurrence and fate of antibiotics in the aquatic environment because it is still unclear if their presence in natural waters (even at sub

therapeutic levels) has contributed to the enhancement of antibiotic resistance amongst aquatic micro-organisms (Martinez, 2009; Baquero et al., 2008).

Several antibiotics are produced by environmental bacteria (e.g. streptomycin) and bacteria with intrinsic resistance to natural antibiotics are found in environmental waters (e.g. sewage, treated effluent and surface waters). However, bacteria with resistance and even multi-resistance to chemically modified and synthetic antibiotics have been found in environmental waters (Watkinson et al., 2007; Ash et al.,2002; Pathak et al., 1993; Jones et al., 1986). Antibiotic resistance is a naturally occurring trait of micro-organisms, however bacteria have developed different mechanisms to render ineffective the antibiotics used against them in order to survive and evolve (Kummerer, 2004). The genes encoding for these different resistance mechanisms are located on bacterial chromosomes and are passed on to the next generation during cell division. In addition, genes encoding for resistance are located on mobile extra chromosomal elements (e.g. plasmids). These extra chromosomal elements, through conjugation, can facilitate the transfer of resistant genes between bacterial species (Wellington et al., 2013)

Wastewater treatment plants (WWTPs) not only receive antibiotic residues following excretion but also faecal bacteria which may harbour resistance genes (Baquero et al., 2008). With the high densities of bacteria in aerobic and anaerobic tanks and the abundance of nutrients in wastewater, WWTPs are probable hotspots for the horizontal gene transfer and therefore the dissemination of antibiotic resistance. This results in a potential impact on the environment when discharged wastewater effluents enter receiving waters or

sewage sludge is used in agriculture (Reinthaler et al., 2009; Zhang et al., 2009; Guardabassi et al., 2002).

Ultimately, the spread of antibiotic pollution due to human perturbations could alter the microbial populations in environmental waters (Martinez, 2009). This could result in resistant bacteria finding their way into drinking waters, giving rise to another potential risk for human health (Watkinson et al., 2007), as the prevalence of antibiotic resistant pathogens increasingly threatens effective management of infectious diseases (Ohlsen et al., 2003).

According to Directive 2001/83/EC on the community code relating to medicinal products for human use (European Commission, 2001), an environmental risk assessment (ERA) must be performed for new pharmaceutical products. Guidelines for the ERA of pharmaceutical products have been created (European Medicines Agency, 2006) and include fate and effect experimental studies (The Organisation for Economic Co-operation and Development, 2013). These studies give valuable information on biodegradability, sorption, bioaccumulation and toxicity. However, they do not provide information on the chronic exposure to environmentally relevant concentrations, such as has been observed with oestrogenic compounds on wild fish (Jobling et al., 1998), or evaluate more specific effects such as the development of antibiotic-resistant bacteria. Few studies have investigated the prevalence of antibiotic resistant bacteria in environmental waters (Faria et al., 2009; Servais et al., 2009; Watkinson et al., 2007) although some have investigated the impact of antibiotic exposure on the resistance rates of aquatic bacteria through the use of laboratory scale experiments (Engemann et al., 2006; Helt et al., 2011). Most of these studies have

defined antibiotic resistance in terms of clinical break points which are used in medicine to assess the likelihood of antibiotic therapy success (Kahlmeter et al., 2003). These breakpoints may not be adequate to detect emerging resistance in environmental bacteria and therefore the development of antibiotic resistance in environmental waters may be misinterpreted or underestimated.

1.1 Thesis objectives

1.1.1 Rationale

More research is necessary to understand the occurrence, removal and fate of pharmaceuticals in wastewater treatment plants and the impact these substances have on surface water receiving treated effluent discharges. The results from this study will provide information of the occurrence and removal of selected pharmaceuticals throughout a large urban wastewater treatment plant in the UK. This information is important for evaluating wastewater treatment processes with regard to their efficiency in removing pharmaceuticals and the potential environmental risk.

There are now recognised experimental studies to assess the fate and effects of pharmaceuticals in the environment. However, they are only required by legislation for new medicinal products and they are not suitable to assess specific effects such as those of antibiotics (Kümmerer, 2009). More research is necessary to observe the occurrence of antibiotic resistance among faecal bacteria within wastewater treatment systems and in surface waters receiving treated effluent. In addition, a more sensitive interpretation of antibiotic susceptibility data is required to detect subtle changes of resistance. The data from this study will provide information that can be used to assess the removal capability of

antibiotic resistant bacteria by a wastewater treatment process and the impact of the process on the expression of antibiotic resistance.

Currently, there are few studies that demonstrate a link between the presence of low concentrations of antibiotics and the prevalence of antibiotic resistant faecal bacteria in surface waters. Further work is required to assess the impact of antibiotic exposure on the prevalence of antibiotic resistant bacteria in surface waters. The results from this study will provide more information on the impact of antibiotic exposure on the resistance rates in *E.coli* in surface waters receiving treated effluent.

1.1.2 Aims

The aims of this study are: to investigate the pathways of selected pharmaceutical compounds (including selected antibiotics) from urban wastewater into receiving surface water, to then investigate and compare the levels of faecal bacteria (more specifically *Escherichia coli* and *Enterococcus faecium*) and their respective resistant proportions in wastewater and receiving surface water and, to assess the changes in the proportion of antibiotic resistant faecal bacteria in surface water exposed to antibiotics within treated effluent discharges.

The specific objectives are:

1. To quantify selected pharmaceuticals in screened sewage, settled sewage, final treated effluent and receiving surface water both up- and down-stream from the treated effluent discharge point using solid phase extraction and liquid

chromatography-mass spectrometry. To assess the presence and removal of these selected compounds during wastewater treatment processes.

2. To confirm and monitor the presence of *Escherichia coli* and *Enterococcus faecium* in settled sewage, final treated effluent and receiving surface water both up- and down-stream of the wastewater treatment plant treated effluent discharge point.
3. To determine antibiotic resistance profiles of *Escherichia coli* and *Enterococcus faecium isolated* from the four sampling points using phenotypic antibiotic susceptibility analysis. To assess the impact of the discharged treated effluent on the proportion of *Escherichia coli* and *Enterococcus faecium* resistant to antibiotics in receiving surface water
4. To better understand the impact of antibiotic exposure on the level of antibiotic resistance among *Escherichia coli* in surface waters receiving treated effluent through the use of microcosm studies.

1.2 Organisation of thesis

This thesis has been divided into 7 chapters. Three chapters incorporate the results and their discussion (Chapters 4-6) and are preceded by three introduction chapters (Chapters 1-3). Following a general introduction and an outline of the aims and objectives (Chapter 1) the first literature review chapter identifies the sources and occurrence of pharmaceuticals in environmental waters (Chapter 2). Also included are the current analytical techniques used to detect PPCPs in environmental waters and the current legislation pertaining to these compounds in the environment. Chapter 3 reviews the literature relating to the use of faecal bacteria as indicators of water quality and the prevalence of antibiotic resistance

among faecal bacteria. Additionally, a review of the techniques available to detect and enumerate faecal bacteria is covered and the methods used for determining antibiotic resistance. Chapter 7 presents the thesis conclusions and recommendations for future research.

Chapter 4 presents the optimisation and development of an analytical method to quantify trace concentrations of selected pharmaceuticals in water samples collected from a large urban wastewater treatment plant and receiving surface waters. The removals of PPCPs are evaluated by determining the concentrations at different stages of their treatment process and the impacts of the discharged effluent on receiving surface waters are assessed by comparing pharmaceutical concentrations in surface waters up- and down-stream of the treated effluent discharge point. Prescription data are used as a tool to predict the influent wastewater treatment plant concentrations which are compared to the measured levels.

Chapter 5 presents the distribution of antibiotic resistant *Escherichia coli* (*E.coli)* and *Enterococcus faecium* (*E.faecium)* at different stages of the wastewater treatment process and within the receiving surface water. The faecal bacteria were isolated and enumerated and tested for antibiotic susceptibility using culturable techniques. Identification of bacteria was performed using phenotypic and mass spectrometry techniques. The definition of resistance in environmental bacteria is discussed and antibiotic susceptibility data are compared to epidemiological cut off values which are a more sensitive measure of detecting emerging resistance.

Chapter 6 presents the changes in the prevalence of ciprofloxacin resistant *E.coli* in surface waters following exposure to an antibiotic within wastewater treated effluent discharges.

This is achieved using laboratory microcosm studies over a 14 day period. Different levels of antibiotic exposure are investigated. A chromogenic medium specific to the detection of *E.coli* is supplemented with the antibiotic as a tool to determine the prevalence of antibiotic resistant *E.coli* within the constructed microcosms.

2 The Occurrence of Pharmaceuticals in Environmental Waters

2.1 Pharmaceuticals

Pharmaceutical products are designed to cure and treat disease, improve health, and increase life span (Cunningham et al., 2009). However, they cannot be categorised as a homogenous group of compounds, since they vary widely in properties such as molecular weight, chemical structure and functionality (Cunningham, 2008). Pharmaceuticals are typically large, chemically complex structures, containing multiple ionisation sites spread throughout the molecule and display a variety of physico-chemical characteristics including acid dissociation constants (pK_a), water solubility and octanol water coefficients (log K_{ow}) (Cunningham, 2008). Pharmaceuticals can be classified according to their purpose and biological activity (e.g. antibiotics, analgesics, lipid regulators, antiepileptic substances, anti-inflammatories, antihistamines and X-ray contrast media etc.) (Kümmerer, 2009b).

2.1.1 Pharmaceutical consumption

Over 3000 different pharmaceuticals are commonly used in Europe and new pharmaceutically active substances are continually being developed and introduced into the market place. The NHS Information Centre - Prescribing and Primary Care Services (2012) reported a 4.8 % increase in the £12.9 billion NHS expenditure on medicines between 2009 and 2010 and Intercontinental Marketing Services (IMS) - Health Market Prognosis (2011) have forecast that global pharmaceutical sales will reach US$1.1 trillion by 2014. The use of these compounds will continue to increase with increasing population size and associated demand.

Consumption patterns vary between different countries and over time, depending on medicinal product regulations and approvals, prescribing practices, population sizes and health care systems (Ternes et al., 2008). It is difficult to obtain a reliable estimate of the quantities of pharmaceuticals used each year and currently in England, a central or regional record of pharmaceutical use in hospitals or in over-the- counter medicines is not readily accessible and therefore it is challenging to investigate consumption patterns. In England, consumption data for prescribed medicinal products can be obtained through the manipulation of prescription cost analysis (PCA) data collated by The Health and Social Care Information Centre - Prescribing and Primary Care Services (2012).

2.2 Sources of pharmaceuticals into the aquatic environment

The major sources of pharmaceuticals in the environment together with their water transport routes are shown in Figure 2-1. Wastewater treatment plants (WWTPs) are major contributors of pharmaceuticals to the environment, mainly through excreta or disposal of unused or expired drugs (Gros et al., 2006). WWTPs are not designed to remove or reduce such compounds and therefore some pharmaceuticals are incompletely removed and are discharged in treated effluents, mainly to our rivers. In addition, direct input into rivers is also possible from storm water overflows and leaks in sewer systems. Pharmaceuticals can also accumulate in sewage sludges and ultimately can be released to the environment through the application of the sludge as an agricultural fertilizer. Furthermore, irrigation of treated effluent on arable land can potentially lead to contamination of groundwater if the pharmaceutical compounds are not easily removed through sorption or degradation processes in soil.

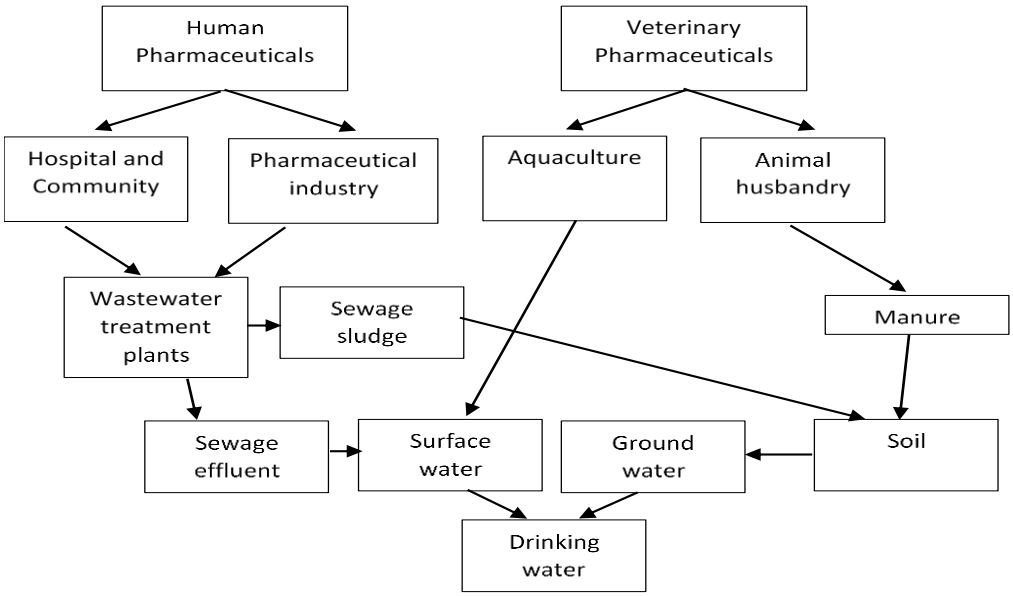


Figure 2-1: Flow chart showing the major pathways of pharmaceuticals within the environment.

In the farming industry, veterinary medicines are widely used to treat disease and protect the health of animals. Release of the parent veterinary pharmaceutical and associated metabolites from animals to the environment can occur directly through excretion on to pasture or application of manure to land. Through leaching or agricultural runoff, these veterinary residues can enter our natural waters (Boxall et al., 2002).

2.2.1 Pharmaceuticals and the wastewater treatment process

2.2.1.1 Wastewater

Wastewater is collected from residential, commercial and industrial establishments. It includes household liquid waste from toilets, baths, showers, kitchens and sinks that is disposed of via sewers. Proper collection, treatment and discharge of waste water, and correct disposal or re-use of the resulting sludge help to protect and improve water quality in the UK. Urban waste water, commonly referred to as sewage, is generally a mixture of

domestic waste water from baths, sinks, washing machines and toilets, waste water from industry and rainwater run-off from roads and other surfaced areas (Maier et al., 2009).

2.2.1.2 The wastewater treatment process

Wastewater treatment can be differentiated into primary, secondary and tertiary stages. In primary treatment, physical operations are used to facilitate the removal of solids. Following the removal of gross solids and a brief residence in a grit chamber to allow sand and grit to settle out, the effluent is transferred into primary settling tanks. Approximately half the suspended solids in wastewater will settle to the bottom of this tank, resulting in the production of a primary sludge. Pathogenic bacteria are not removed effectively during this treatment step, although some sedimentation of these species does occur (Defra, 2002).

In secondary treatment, biological and chemical operations are used to reduce organic matter. The settled sewage undergoes biological treatment in which the remaining organic suspended solids together with soluble organics are biodegraded by aerobic organisms. This treatment step has a large (biochemical) biological oxygen demand and therefore a continuous air supply needs to be maintained. Secondary biological treatment is achieved using either trickling filter beds or (conventional) activated sludge tanks.

Trickling filter bed treatment involves spraying the settled sewage over a substrate composed of plastic units (in older plants, the filter is a bed of stones) coated with a micro-organism biofilm. The spraying process oxygenates the settled sewage and facilitates the aerobes to decompose the organics. In the activated sludge process, settled sewage is transferred to an aeration tank, agitated, aerated and mixed with a bacteria rich sludge remaining from earlier treatment (activated sludge) to encourage decomposition of the

remaining organic material. From the aeration tank, the effluent moves to a sedimentation tank to allow microbial flocs to settle. Important parameters that need to be controlled during the activated sludge process to ensure effective treatment include the hydraulic retention time (typically four to eight hours), the food to micro-organism ratio (organic load to micro-organisms expressed as BOD/kg) and the oxygen supply rate. Both the aeration process and secondary sedimentation contribute to the inactivation or removal of pathogenic bacteria by microbial antagonism or by floc formation in which the pathogens may be trapped and settle out.

Sometimes further treatment (tertiary) is required to protect sensitive water environments (Defra, 2012). Tertiary treatment is practised to further reduce nutrients such as nitrogen and phosphorus, metals and organics (Guardabassi et al., 2002) to provide additional protection of the environment after effluent discharge into rivers or lakes. It is also performed when effluent is to be used for irrigation (e.g. food crops) or as a drinking water source. Tertiary treatment is expensive and is usually reserved for the discharge of treated effluents into sensitive areas (e.g. eutrophic waters) and bathing waters to ensure compliance with legislation (e.g. Urban Wastewater Treatment Directive). Tertiary treatments include filtration, sorption to activated carbon, disinfection, ozonation and UV oxidation.

2.2.1.3 Removal of pharmaceutical active compounds in wastewater treatment processes.

The main aims of the wastewater treatment process are to:

- reduce the organic content of wastewater including toxic or recalcitrant trace organic compounds
- reduce suspended solids
- reduce or inactivate pathogenic bacteria
- reduce the nutrient loads discharged to receiving surface waters

It has been reported that wastewater treatment plants are inefficient at eliminating pharmaceutical compounds resulting in the discharge of these compounds to receiving surface waters (Radjenovic et al., 2009). In addition, the removal efficiencies of these compounds from wastewater vary considerably between different treatment plants (Farré et al., 2007; Quintana et al., 2005; Miao et al., 2004; Petrovic et al., 2003; Golet et al., 2001). Longer solid retention times and hydraulic retention times contribute to higher removal efficiencies (Ternes et al., 2008; Ternes et al., 2004; Kummerer, 2003) and significantly higher removal rates were observed for antibiotics (Göbel et al., 2005) and pharmaceuticals (Clara et al., 2005) with increases in sludge age.

The primary pharmaceutical removal mechanisms associated with biological wastewater treatment processes are sorption and biological transformation (Jelic et al., 2011). Other removal mechanisms include stripping due to aeration or photodegradation but these are considered to be non-existent or have a negligible effect (Ternes et al., 2008).

Pharmaceuticals can sorb to particulate matter which facilitates their removal by settling or flotation. Sorption depends on two main mechanisms:

1. Absorption through hydrophobic interactions between aliphatic or aromatic functional groups of the pharmaceutical compound with the lipid fractions of suspended solids
2. Adsorption through electrostatic interactions of positively charged functional groups of the pharmaceutical compound with negatively charged surfaces of microoganisms.

Several approaches have been used to determine the affinity of a given substance to solids. The tendency of a chemical to sorb and accumulate in solids can be assessed by the octanol-water partition coefficient (K_{ow}) or the organic carbon-based coefficient (K_{oc}) (Carballa et al. (2008). However, they are both more useful for investigating the sorption of uncharged molecules where the interactions are mainly hydrophobic in nature. The determination of the sorption coefficient (K_d) is more useful for predicting the potential of a pharmaceutical compound to sorb to wastewater solids (Carballa et al. (2008). The sorption coefficient K_d is used to describe the solid liquid partitioning characteristics of a compound and this value is the ratio of the sorbed phase concentration to the solution phase concentration at equilibrium as shown in Equation 2-1 (Ternes et al., 2008). The removal of active compounds through sorption mechanisms is considered negligible (< 10 %) for compounds with K_d values $\leq$ 300 L/kg (Ternes et al., 2008).

Equation 2-1:

$$K_d = \frac{X_{part}}{S} = \frac{X}{X_{ss} \times S}$$

Where:

X concentration sorbed onto sludge per unit reactor volume (μg/L)

X_{part} concentration sorbed, per amount of sludge dry matter (μg/g)

K_d solid-water distribution coefficient (L/g)

X_{ss} suspended solids concentration in raw water or production of suspended solids in primary and or secondary treatment per L of wastewater (g/L)

S dissolved concentration (μg/L)

The biological transformations of pharmaceutical active compounds during the wastewater treatment process include; mineralisation, transformation to more hydrophobic compounds which adsorb to solid particles, and transformation to more hydrophilic compounds which remain in the liquid phase. The degree of biodegradation will depend on the characteristics of the active compound and on the wastewater treatment plant operating conditions including the biodiversity of the microbial biomass, the floc size of the sludge, the fraction of the active biomass within the total suspended solids and temperature (Ternes et al., 2008; Clara et al., 2005). There are now increasing reports of biodegradation studies, however some only report the degradation of the original active compound but do not investigate the appearance of metabolites or transformation products which may also have an ecotoxicological effect (Alexy et al., 2004; Kümmerer et al., 2000; Al-Ahmad et al., 1999).

Tertiary wastewater treatment processes (e.g. sand filtration and disinfection) and advanced UV and ozonation processes have been assessed for the elimination of pharmaceuticals from wastewaters (Quintana et al., 2009; Sharma, 2008; Hua et al., 2006; Andreozzi et al., 2003a). High removal rates have been achieved with advanced processes,

however the formation of by products and their respective ecotoxicity are not always known (Senta et al., 2011; Sharma, 2008).

2.3 Occurrence of pharmaceuticals in wastewater and surface water

There are numerous reports describing the presence of pharmaceuticals in wastewater influents, effluent and surface waters. Table 2-1 lists the occurrence of some pharmaceuticals from different classes (e.g. antibiotics, anti-inflammatories, beta-blockers, anticonvulsants and lipid regulators) in environmental waters. The environmental fate of pharmaceutical actives in aquatic environments will depend on the different physico-chemical properties of these substances, environmental conditions, the wastewater treatment employed and consumption levels within the catchment area (Cunningham, 2008). These factors represent a challenge when estimating the loads of these substances in aquatic environments and justify the variety in pharmaceutical concentrations which have been reported in environmental waters.

The presence of pharmaceuticals in wastewater effluents and rivers confirms that municipal wastewater treatment plants do not completely remove these compounds. Some pharmaceuticals, such as carbamazepine, have been frequently reported in environmental waters leading to its proposal as an anthropogenic marker for the aquatic environment (Clara et al., 2004). Conversely, vancomycin is rarely reported and possibly this could be attributed to inadequate analytical methods or to low consumption patterns. In a study reported by Sim et al. (2011), carbamazepine was detected in 100 % of the sampled municipal wastewater influents and effluents , whilst vancomycin was not detected at all.

Table 2-1: Pharmaceutical compounds detected in wastewater influents, effluents and surface waters

Compound	Sample matrix	Concentration (ng/L)	Location	Reference
Sulfasalazine Amoxicillin Ibuprofen Diclofenac Carbamazepine Bezafibrate	Wastewater influent	65 ND 3742 70 2593 971	UK	Kasprzyk-Hordern et al. (2008)
Diclofenac Bezafibrate Carbamazepine Clarithromycin	Wastewater influent	400-1500 400-1400 400-1400 400-1000	Spain	Jelic et al. (2011)
Carbamazepine, Clarithromycin, Chlortetracycline Ciprofloxacin, Diclofenac, Sulfonamides Tetracycline	Wastewater influent	24.8-50.9 ND-724.2 ND- 15.9 11.4-300.7 ND–9.5 ND-261 ND-38.9	US	Spongberg et al. (2008)
Carbamazepine Ciprofloxacin Vancomycin Diclofenac Penicillin G	Wastewater influent	95-21600 124-246 ND 94-523 ND	Korea	Sim et al. (2011)
Clofibric acid Ketoprofen Ibuprofen Diclofenac Carbamazepine	Wastewater effluent	ND-90 330-700 ND-34 ND-30 290-960	Taiwan	Chen et al. (2008)
Bezafibrate Ibuprofen Diclofenac Carbamazepine Ciprofloxacin	Wastewater effluents	ND-1007 50-7110 680-5450 870-1200 60-70	France, Greece, Italy, Sweden	Andreozzi et al. (2003b)
Acebutolol Metoprolol Carbamazepine Ciprofloxacin	Wastewater effluent	390-510 980-1350 290-400 200-650	Finland	Vieno et al. (2006)
Amoxicillin Chloramphenicol	River water	ND 266	Hong Kong	Xu et al. (2007)
Carbamazepine Ibuprofen Codeine	River water	65.4-75.1 61.3-115.2 26.6-53.6	Romania	Moldovan (2006)
Bezafibrate Carbamazepine Clarithromycin Ibuprofen	River water	<50-130 <30-140 <30-40 <20-70	Germany	Wiegel et al. (2004)

ND = not detected

2.4 Analytical methods to detect pharmaceuticals in environmental waters

2.4.1 Sample preparation and analysis

Environmental waters are complex matrices and pharmaceuticals are typically present at only trace levels (ng/L). Therefore, analytical methods require efficient sample extraction and pre-concentration procedures to achieve the desired level of analytical sensitivity and selectivity. Sample extraction and pre-concentration are usually achieved by using solid phase extraction (SPE) methods (Gros et al., 2006; Miao et al., 2002). SPE involves passing the water sample through a cartridge containing a sorbent known to retain the compounds of interest but to release other compounds that can interfere with or suppress the detection signal. The retained compounds are subsequently desorbed and eluted from the sorbent with appropriately selected organic solvents. The volume of eluate is reduced under nitrogen and reconstituted with a small volume of a solvent suitable for the chosen analytical technique. A range of SPE sorbents is available employing different retention mechanisms based on hydrophobic interactions, dipole/dipole interactions, and/or ion exchange. Many methods utilise copolymer or polymeric sorbents (Tong et al., 2009; Gros et al., 2008) which provide more than one retention mechanism and are useful for the extraction and pre-concentration of polar and moderately polar analytes. Other extraction methods that have been used to clean up and pre-concentrate pharmaceuticals from environmental waters include hollow fibre-based liquid phase microextraction (HF-LPME) (Payán et al., 2010) and solid-phase microextraction (SPME) (Sanchez-Prado et al., 2006) but these are less common.

Different analytical techniques have been employed for the detection of pharmaceuticals in environmental samples including capillary electrophoresis (Nozal et al., 2004), high performance liquid chromatography with ultra-violet detection (HPLC-UV) (Sturini et al., 2009; Benito-Peña et al., 2006), high performance liquid chromatography with fluorescence detection (HPLC-Fl) (Golet et al., 2001) and gas chromatography-mass spectrometry (GC-MS) (Hao et al., 2007). The most common analytical method used in this field is liquid chromatography-mass spectrometry (LC-MS^n) (Hernández et al., 2007). LC-MS^n is a sophisticated technique that is suitable for the separation and analysis of non-volatile compounds with medium to high polarity, such as pharmaceuticals. In comparison to techniques such as GC-MS (suitable for volatile compounds) it does not require derivatization steps prior to analysis and has advantages over HPLC-UV in that it provides compound confirmation. Liquid chromatography coupled with tandem mass spectrometry (LC-MS^2) permits improved sensitivity compared to single quadruple mass spectrometry (Pérez et al., 2007).

2.4.2 Liquid chromatography-mass spectrometry (LC-MS^n)

LC-MS^n couples high performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) with mass spectrometry detection typically using either an electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) interface. HPLC is used to separate sample components by using their difference in partitioning behaviour between the stationary phase (HPLC column) and mobile liquid phase (solvent). The separated components are then ionised before transmission to the mass analyser. Ionisation occurs within the interface. With electrospray ionisation, samples are ionised by

applying a high voltage to the HPLC eluant after conversion to a heated spray using a nebulising gas (usually nitrogen). The resulting gas phase ions are focused through the mass analyser to achieve separation according to their mass to charge ratio (*m/z*). Electrospray ionisation is a soft ionisation technique which results in very little fragmentation and therefore produces molecular ions (deprotonated or protonated) leading to molecular weight information (Seifrtová et al., 2009).

The quadrupole analyser is the most common mass analyser used in LC-MS^n systems and uses the stability of ion trajectories in oscillating electric fields to separate the ions according to their *m/z* value. Quadrupole analysers consist of four parallel metal rods where adjacent rods have opposite voltage polarity applied to them. The electric force on the ions causes them to oscillate/orbit in the area between the four rods and therefore affects the trajectories of the ions focussed into the analyser (Figure 2-2).

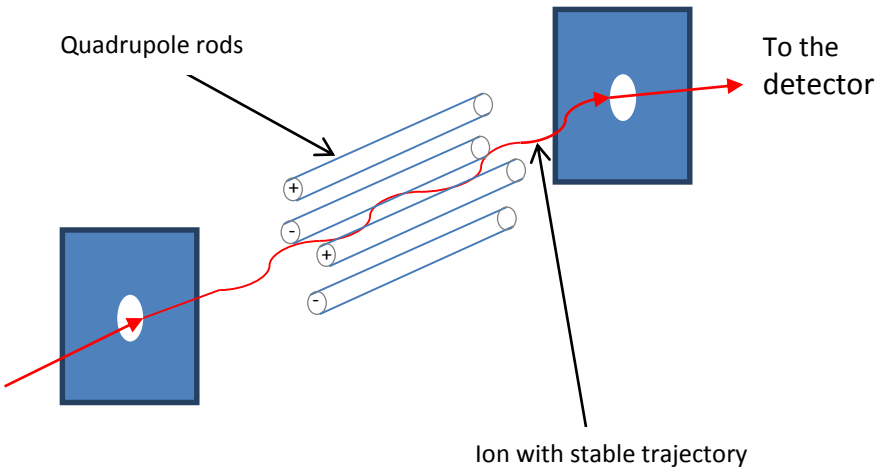


Figure 2-2: Schematic showing ion trajectories through a quadrupole *m/z* selector

To ensure the uninhibited movement of the gas-phase ions within the instrument, a vacuum is generated using rotary and turbo-molecular vacuum pumps. Before entering the quadrupole, the ions travel through a potential of a certain voltage, generated by a ring electrode, in order to give the ions a constant velocity so they can transverse along the centre of the quadrupole. The ion moves in a very complex motion that is directly proportional to the mass of the ion, voltage on the quadrupole, and the radio frequency. Only stable ions pass in between the rods to the detector and are then counted by striking the ion detector, generating an amplified signal which is sent to the computer for data processing.

2.5 Legislation relating to the occurrence of pharmaceuticals in environmental waters

There are a number of European directives and regulations including the Water Framework Directive (WFD), the Urban Waste Water Treatment Directive (UWWTD) and the Registration, Evaluation, Authorisation and restriction of Chemicals regulation (REACH) which are designed to protect our natural waters from the adverse effects of chemical pollutants and wastewater discharges. Currently, there is no requirement for the monitoring of pharmaceutical active compounds in natural waters, despite the concerns from the scientific community. However, it is now mandatory to carry out an environmental risk assessment of new medicinal products according to Directive 2001/83/EC on the community code relating to medicinal products for human use (European Commission, 2001).

2.5.1 The Water Framework Directive

The Water Framework Directive (WFD, 2000) was implemented to regulate how water bodies (surface waters including, rivers, lakes, and coastal waters) are managed throughout Europe to prevent further deterioration of aquatic ecosystems and to reduce pollution especially by harmful substances listed in the daughter directive - Environmental Quality Standards Directive (European Commission, 2008). There are 41 substances currently listed in this Directive although they do not include pharmaceutical compounds, despite 4 yearly reviews and proposals to include pharmaceuticals such as diclofenac.

2.5.2 Urban Wastewater Treatment Directive (UWWTD)

The Urban Wastewater Treatment Directive (UWWTD, 1991) regulates how wastewater is collected and treated from domestic and industrial sources. It is designed to protect our environment from the adverse effects that untreated sewage can have and is imperative for protecting public health. The Directive identifies the conditions that should be met before treated effluents are discharged to receiving waters. These include the specified limits prior to discharge of (biochemical) biological oxygen demand, chemical oxygen demand, suspended solids and total phosphorus and nitrogen which all should be reduced to specified limits before discharge. Reduction of emerging pollutants such as pharmaceuticals are not included in the Directive and therefore monitoring of these compounds in the effluents is not enforced.

2.5.3 Registration, evaluation, authorisation and restriction of chemicals (REACH)

REACH is a European Union regulation designed to protect human health and the environment from the use of chemicals (European Commission, 2006). The regulation requires that manufacturers and importers of chemicals are responsible for the understanding and management of the risks associated with those chemicals. However, human and veterinary pharmaceuticals are not covered by REACH as they are covered by EU pharmaceutical legislation (European Commission, 2001).

2.5.4 European Medicines Agency (EMA)

Directive 2001/83/EC on the community code relating to medicinal products for human use (European Commission, 2001) states that an evaluation of the potential environmental risks must be undertaken for new drugs coming to market. A guidance document (European Medicines Agency, 2006) details a two phase approach for conducting an environmental risk assessment (ERA). In phase I (the exposure assessment), the maximum predicted concentration expected in surface waters ($PEC_{surfacewater}$) from the discharges of wastewater treatment plants is estimated. If the concentration is estimated to be less than the action limit (10 ng/L), the ERA may be terminated at this step. In cases where the predicted environmental concentrations exceed the action limit, the second step (Phase II) is recommended.

In Phase II, experimental studies are required to assess the fate and effects of the pharmaceutical compound under test to determine hazard quotients (Ginebreda et al., 2010). Physico-chemical characteristics of the test compound including the octanol water coefficient (K_{ow}), which is used as an indicator for bioaccumulation, and the soil organic

carbon-water partitioning coefficient (K_{oc}), which is recommended to describe the sorption behaviour of the pharmaceutical compound in sewage sludges are also evaluated. An octanol water coefficient value > 1000 indicates the pharmaceutical can bioaccumulate in aquatic organisms. A soil organic carbon-water partition coefficient value > 10,000 L/kg indicates the pharmaceutical can be retained in the sewage sludge and therefore possibly eventually transported to the terrestrial environment through land spreading (European Medicines Agency (2006). The experimental studies recommended to assess the physico-chemical properties, fate and effects of pharmaceuticals are given in Table 2-2 and are reported by The Organisation for Economic Co-operation and Development (2013).

The effect studies use a set of organisms representing the aquatic ecosystem and food chain web to test for acute and chronic toxicity. From this data and with the use of an assessment factor (AF), the predicted no-effect concentration ($PNEC_{surfacewater}$) is determined which is used for estimating hazard quotients and therefore risk characterisation. The assessment factor is used to account for the degree of uncertainty involved in extrapolating laboratory study data to the real environment, inter species variations and differences in sensitivity and intra species variability (Ternes et al., 2008). The hazard quotients are defined as the ratio between the pharmaceutical predicted surface water concentrations ($PEC_{surfacewater}$) and the predicted no-effect concentrations ($PNEC_{surfacewater}$). Under this environmental risk assessment (ERA) structure, an unacceptable environmental risk is indicated if the hazard quotients are > 1.

Table 2-2: Physico-chemical, fate and effect studies recommended for the environmental risk assessment of new medicinal products with predicted surface water concentrations exceeding 10 ng/L.

Experimental study	Recommended test protocol
Adsorption-desorption batch equilibrium method	OECD 106/OECD121
Ready biodegradability	OECD 301
Aerobic and anaerobic transformation in aquatic sediment systems	OECD 308
Algae, growth inhibition test	OECD 201
Daphnia sp. Reproduction test	OECD 211
Fish early life stage toxicity test	OECD 210
Activated sludge respiration inhibition test	OECD 209

Taken from European Medicines Agency (2006). OECD – the organisation for economic Co-operation and development.

This approach to ERA provides valuable information. However, the outcome does not constitute a reason to prevent a new drug being authorised for sale as the human medical benefits have precedence over any environmental risk (European Medicines Agency, 2006). However, if an environmental risk is identified, there are recommendations for restricted use (e.g. hospital only) and product labelling to ensure correct disposal. Currently there is no legislation relating to drugs already available on the market, unless an application for authorisation to change dose or application is submitted. For veterinary medicines, the outcome of the environmental risk assessment may serve as a basis for minimising the quantity of the medicine released into the environment.

3 Water quality indicator bacteria and antibiotic resistance

Natural waters polluted by faecal contamination from humans and animals transport a variety of human pathogenic microorganisms (viruses, protozoa and bacteria). The detection of waterborne pathogens is difficult and therefore various indicators of faecal contamination are used to detect faecal pollution (Servais et al., 2009). Human commensal and pathogenic bacteria are constantly released with wastewater to natural waters. A proportion of these organisms will be resistant to antibiotics. It is believed that the continuous release of low levels of antibiotics and resistant bacteria with wastewater has the potential to enhance the dissemination of antibiotic resistance in environmental bacteria (Castiglioni et al., 2008).

3.1 Microbial indicators of water quality

The use of indicator organisms as a means of assessing the potential presence of water-borne pathogens through the use of simple microbiological tests has been paramount to protecting public health. Indicator organisms are selected bacteria that when present in water are indicative of either faecal contamination or deterioration of water quality (Environment Agency, 2002).

The criteria for an ideal indicator organism include the following:

- The organism should be a member of the intestinal microflora of warm blooded animals
- The organism should be present when pathogens are present

- The organism should be present in greater concentrations than the pathogen
- The organism should survive longer than the hardiest of pathogens
- The organism should not grow and multiply in water
- The organism should be easily detectable by inexpensive methods
- The concentration of the organism should relate to the degree of faecal pollution

There is no one indicator organism that fulfils all of the criteria and therefore various groups of microorganisms have been suggested and used as indicators for example coliforms, *Escherichia coli*, enterococci, pseudomonas *aeruginosa* and *Staphylococcus aureus*.

3.1.1 Coliform bacteria and Escherichia coli

The coliform group belong to the family Enterobacteriaceae. Typical genera encountered in water supplies are *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia* and *Yersinia* (Environment Agency, 2002). Coliform bacteria are used as an indicator of faecal pollution because some species originate from faecal sources, survive longer than some pathogenic bacteria and are easy to detect and enumerate. However, coliform bacteria can grow in natural waters in appropriate conditions (depending on the amount of organic matter and temperature) and can therefore give a false indication of faecal pollution (Maier et al., 2009; Bitton, 1994). In addition, coliform bacteria are less resistant to disinfectants than protozoans and viruses. Therefore their usefulness as an indicator is limited (Maier et al., 2009; Bitton, 1994). Coliforms are aerobic, facultative anaerobic, gram-negative, non-spore forming, rod shaped bacteria that produce gas due to lactose fermentation in culture media within 48 hrs at 35 °C. They do not produce cytochrome C oxidase and are therefore oxidase negative (Environment Agency, 2009). Coliforms include all coliforms that can

ferment lactose at 44 °C. Not all coliform bacteria are exclusively of faecal origin except for *E.coli*. In human excreta, the average density of faecal coliforms per gram is 10^7 and for animals, the average density of faecal coliforms per gram of faeces can be in the range of 10^1 to 10^7 depending on the animal taxonomy (Maier et al., 2009). Whilst coliform bacteria are used to indicate the presence of other pathogens, they can themselves be responsible for causing infection and illness. Coliform species include Enterobacter species (*E. cloacae and E. aerogenes)* and are commonly identified as the cause of urinary and respiratory tract infections. In addition, some *Klebsiella* species are also opportunistic pathogens. *K. pneumoniae* is the most frequently isolated *Klebsiella* species from wound, bloodstream and urinary tract infections (Health Protection Agency, 2008). However, *E.coli* is the most frequent cause of urinary tract and kidney infections and is the most important food poisoning pathogen worldwide. Some strains of *E.coli* such as *E.coli* O157:H7 cause disease by producing a toxin called Shiga toxin. In addition, *E.coli* are opportunistic pathogens that can cause disease and are increasingly responsible for bloodstream infections (bacteriamas) in the UK (Health Protection Agency, 2007).

3.1.2 Enterococci

Enterococci are gram-positive aerobic, facultative anaerobic, non-spore forming cocci and can be distinguished from closely related bacteria by their ability to grow in 6.5 % NaCl, at a pH of 9.6 and a temperature of 45 °C (Environment Agency, 2002). The species of enterococci that occur in faeces and, therefore, are more likely to be found in polluted waters include *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus hirae*. Enterococci are used as indicator organisms because they survive environmental stress longer than coliforms and therefore many pathogenic bacteria, rarely multiply in

environmental waters but persist for long times. However, they are not exclusively from the faeces of humans (Junco et al., 2001). In human faeces the average density of enterococci per gram of faeces is in the region of 10^6 whereas, in animals the average concentration of enterococci varies according to animal type (domestic, wild or farm animal) and can be in the range of 10^4 to 10^6 with considerable variation between species (Maier et al., 2009; Anderson et al., 1997). Enterococci can cause infections in humans including urinary tract infections, bacteraemia (blood stream infections) and wound infections. However, 95% of enterococci infections are caused by two enterococci species, *Enterococcus faecium* and *Enterococcus faecalis* (Helt et al., 2012). Enterococci are resistant to many antibiotics, so infections are most commonly seen in patients hospitalised for long periods of time and receiving broad spectrum antibiotics.

3.1.3 Staphylococci

Staphylococci are gram-positive non-spore forming, non-motile, aerobic, facultative anaerobic cocci bacteria that produce catalase (from hydrogen peroxide) and have the ability to grow in 6.5 % NaCl. Staphylococci are ubiquitous in the environment yet are not always of faecal origin. However, they have been advocated as indicator organisms of water quality in recreational waters and where appropriate, provide a measure of effective water treatment and disinfection (Environment Agency, 2000). Staphylococci are mainly associated with the skin, respiratory tract and gastrointestinal tract of humans and warm-blooded animals and readily gain access to water when a body is immersed. *Staphylococcus aureus* is a pathogenic organism causing wound, skin infections and urinary tract infections and can be differentiated from other staphylococci species based on its ability to produce coagulase whilst typically other species cannot. The density of coagulase positive

staphylococci in raw sewage has been estimated at approximately 10^3 CFU/100 mL (Maier et al., 2009).

3.1.4 Pseudomonas aeruginosa

Pseudomonas aeruginosa are Gram-negative, oxidase-positive bacteria which usually produce pyocyanin and fluorescein pigments (Environment Agency, 2010). *Pseudomonas aeruginosa* are frequently present, in small numbers, in the normal intestinal flora of humans and animals and can be present in raw sewage at concentrations of 10^5 CFU/100 mL (Maier et al., 2009). However these organisms should not be used as an indicator of faecal pollution as they are commonly found in soil and on plants and are able to grow in oligotrophic waters (Maier et al., 2009). *Pseudomonas aeruginosa* are opportunistic pathogens and large numbers growing in bathing waters, swimming pool waters or spa pool waters can result in ear infections for those immersed in the polluted waters. Therefore this species is often used as an indicator for recreational water quality (Environment Agency, 2004).

3.2 Detection, enumeration and identification of indicator bacteria

Coliforms (including *E.coli*), enterococci, staphylococci (including *staphylococcus aureus*) and *pseudomonas aeruginosa* can be detected and enumerated in environmental waters by simple bacteriological methods such as the membrane filtration (MF) or the most probable number test (MPN). The significance of the membrane filtration and most probable number tests and the interpretation of results are well authenticated and have been used as a basis for standards of bacteriological quality for environmental waters (Environment Agency, 2000). Some methods are routinely used such as those identified by the Environment

Agency (Environment Agency, 2010; Environment Agency, 2007) or as detailed in Standard Methods for the Examination of Water and Wastewater (Standard Methods Committee (SMC), 2006). Methods employ culture media containing components that encourage the growth of target bacteria whilst inhibiting non target bacteria. In other studies, chromogenic agars have been employed for the selective and differential detection of *Escherichia coli* and coliform bacteria in environmental waters (Wohlsen, 2011; Watkinson et al., 2007; Alonso et al., 1996). Chromogenic agars contain chromogenic enzyme substrates that detect specific enzyme activity characteristic of certain bacterial groups or species. Examples of culture media for the detection and enumeration of Coliforms, enterococci, staphylococci and *pseudomonas aeruginosa* are presented in Table 3-1.

The detection of target bacteria using culture methods are considered presumptive until further confirmation tests have been performed. In addition, the culture media typically used are not always species specific and therefore to identify to species level further identification tests are required using either, biochemical analysis, genomic analysis or even mass spectrometric techniques.

3.2.1 Enumeration methods

The most probable number test (MPN) is useful for the determination of the organisms under test from water samples with high turbidity which may interfere with accurate colony counts (Environment Agency, 2000). In the multiple-tube method, a series of tubes containing a suitable selective broth culture medium is inoculated with different dilutions of a water sample.

Table 3-1: Specific culture media used to detect and enumerate bacteria indicators in environmental waters

Target bacteria	Culture media	Selective/indicating components	Reference
E.coli/coliform	*E.coli/coliform* selective chromogenic agar	• Rose-Gal chromogenic agent: to detect ß-galactosidase enzymatic activity characteristic of *E.coli* but not other coliform bacteria • X-Glu chromogenic agent: to detect ß-glucuronidase activity (fermentation of lactose) characteristic of coliform bacteria. • sodium lauryl sulphate: to inhibit non-target organisms	Wohlsen (2011)
E.coli	Typtone bile x glucuronide agar	• 5-bromo-4-chloro-3-indoyl-β-G-glucuronide to detect ß-glucuronidase activity	Geissler et al. (2000)
Enterococci	Slanetz and Bartley agar	• sodium azide: to inhibit Gram negative bacteria • triphenyltetrazolium chloride: reduced by *enterococci* to produce maroon colonies	Environment Agency (2010a)
Staphylococci (including *Staphylococcus aureus*)	Mannitol salt agar with 0.005 % sodium azide	• Sodium azide and 7.5 % sodium chloride: to supress non-target bacteria • Mannitol: to differentiate between S*taphylococcus aureus* and other staphylococci species • Phenol red: to indicate mannitol has been fermented by turning the red medium yellow	Health Protection Agency (2004)
Pseudomonas aeruginosa	Pseudomonas selective agar	• Magnesium chloride and potassium sulphate: To enhance the pigment production characteristic of *Pseudomonas aeruginosa* • Antibiotics cetrimide and nalidixic acid: to inhibit non-target bacteria	Environment Agency (2010b)

Following incubation, an estimation of the number of bacteria under investigation per unit volume of the original sample can be made from the tubes that give a positive result and by using a standardised MPN table which is based on statistical probabilities (Environment Agency, 2009). The principle of the MPN method is to dilute the sample, so that there will be tubes with and without viable organisms. The MPN test is based on the assumption that all inoculated tubes containing at least viable organisms will produce detectable growth or change (US Food and Drug Administration, 2010).

The membrane filtration method involves passing a known amount of sample (usually 100 mL) through a membrane filter (pore size 0.45 µm) to trap bacteria on the surface. This membrane is then placed on a specific medium that permits the growth of the bacteria of interest and inhibits or differentiates bacteria which are not of interest (Black, 1996). The counts on membrane filters are subject to statistical variation, and replicate tests on subsamples from the same bulk sample are unlikely to give exactly the same number of colonies (Environment Agency, 2000). It has been recommended to report colony counts from filters with approximately between 10 and 100 colonies to minimise statistical errors (Environment Agency, 2007a). An advantage of the membrane filtration technique is that there is considerable saving in labour and in the amount of media and glassware required when compared to traditional most probable number (MPN) techniques (Environment Agency, 2000).

3.2.1.1 Confirmation tests

Enumeration methods use growth media to facilitate the growth of the bacteria of interest. However, some non-target bacteria can cause false positive results and therefore all results

are considered presumptive. Presumptive bacteria can be confirmed through a number of biochemical tests used to detect characteristics specific to the target bacterial group of interest (e.g. coliforms). An example of the biochemical confirmation tests used to confirm presumptive coliforms (including *E.coli*), enterococci, staphylococci and *Pseudomonas aeruginosa* bacteria are presented in Table 3-2.

3.2.2 Identification of bacteria

A range of physiological, serological, biochemical and genomic methods such as 16S rRNA gene sequence analysis, real-time polymerase chain reaction (PCR) assays, and peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH) are typically applied for the identification of bacteria. More recently, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) has emerged as a new technology for species identification (Bizzini et al., 2011).

Traditional methods of bacterial identification rely on phenotypic identification using gram staining, culture and biochemical tests that identify specific metabolic activity (Maier et al., 2009). If the identification to species level is necessary a multitude of tests may be required and therefore phenotypic identification can be very labour intensive. However, there are commercial kits available standardising the biochemical identification process.

Phenotypic methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated in vitro. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species (Winston et al., 2004). To overcome these drawbacks, genotypic identification methods have become widely used.

Table 3-2: Biochemical confirmation tests for indicator bacteria

Confirmation test	Nature of test	Inference	reference
Oxidase	To detect cytochrome oxidase activity using detection strips impregnated with NNN'N' tetramethyl -p- phenylene-diamine dihydrochloride	Cytochrome oxidase activity is characteristic of *pseudomonas aeruginosa*	Environment Agency (2000)
Catalase	To detect the presence of catalase enzymes using hydrogen peroxide. The reaction is detected by the release of oxygen gas bubbles from the decomposition of hydrogen peroxide	staphylococci possess the catalase enzyme	Health Protection Agency (2004)
Indole production	To detect the presence of enzymes that produce indole from the hydrolysis the amino acid tryptophan	Conversion of tryptophan to indole is characteristic of *E.coli* and not other bacteria in the coliform group	Environment Agency (2009)
Gram staining	To distinguish between the cell wall structure of Gram positive and Gram negative bacteria using crystal violet stain.	Gram positive bacteria (e.g. enterococci *and* staphylococci) can retain the crystal violet stain whilst Gram negative (e.g. *E.coli*) cannot.	Maier et al. (2009)
Aesculin hydrolysis	To detect bacteria that can hydrolyse aesculin	Enterococci can hydrolyse aesculin	Environment Agency (2010a)
Growth in 6.5 % sodium chloride	To distinguish between bacteria tolerant to elevated salt levels.	Enterococci can tolerate 6.5 % sodium chloride growth conditions whilst other bacteria closely related to enterococci cannot (e.g. streptococci)	Environment Agency (2010a)

Currently, sequencing of the 16S rRNA gene is accepted as the reference method for bacterial species identification. Although, not perfect, genotypic identification of microorganisms by 16S rRNA gene sequencing has emerged as a more accurate and reliable method for bacterial identification compared to phenotypic techniques, with the added capability of defining taxonomical relationships among bacteria. The difficulties encountered with this technique include the recognition of novel taxa, too few sequences deposited in nucleotide databases, species sharing similar and or identical 16S rRNA sequences (Janda et al., 2007). In addition, it has been reported that this technique is timely and costly, requiring intricate instrumentation and skilled personnel (Bizzini et al., 2011).

Recently, bacteriologists have focused their attention on the use matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) and this recent technique is being increasingly used for more routine purposes (Carbonnelle et al., 2011; Jamal et al. , 2011; Dingle et al., 2009; Eigner et al., 2009). The method analyses bacterial proteins from bacterial cell extracts and provides a unique mass spectral fingerprint of the microorganisms that can be compared to those in a reference database. This new proteomic approach allows rapid and cost effective accurate identification of bacteria.

3.2.2.1 Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS)

Bacteria identification using MALDI-TOF-MS begins by applying a sample of a fresh purified culture (either in a solid or liquid form) onto a defined indentation (well) on a solid target support plate which is then overlaid with a chemical matrix. The prepared target plate is placed into an ionization chamber where each bacterial sample is irradiated with pulses of

energy from a laser (typically an ultra violet nitrogen laser at a wavelength of 337 nm). Although the mechanism of ionisation remains uncertain, it is believed that this process desorbs individual sample and matrix molecules from the target plate into the gas phase, with the majority of energy being absorbed by the matrix, which becomes ionized with a single positive charge. This positive charge is subsequently transferred from the matrix to the sample compounds through their random collision in the gas phase (Kafka et al., 2011). Matrix assisted laser desorption is a soft ionisation technique suitable for the ionisation of large non-volatile compounds such as proteins (Vargha et al., 2006). The matrix is essential for the soft ionization process. It is chosen for its ability to effectively absorb the majority of the ionizing energy thereby protecting the sample molecules from fragmenting. Common matrices for the ionisation of proteins are α-cyano-4-hydroxy-cinnamic acid (HCCA), 2,5-dihydroxybenzoic acid (DHB) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic) (Vargha et al., 2006).

The time of flight (TOF) analyser measures the time it takes for the ionised compounds to travel a fixed distance. A cloud of ionized compounds (e.g. proteins) from each pulse during the ionisation phase are accelerated through a positively charged, electrostatic field into the time of flight (TOF) tube. The TOF tube is a pressurised tube that allows ions to travel down a field-free region toward the ion detector. The velocity at which individual ions travel through the TOF tube is dependent on their mass-to-charge ratio (*m/z*) and therefore ions are separated based on their difference in mass (Kafka et al., 2011). Heavier ions will travel through the mass analyser at a slower velocity, compared to lighter ions. As the ions emerge from the TOF mass analyser, they collide with the ion detector, which measures their charge

and time to impact. Based on standards of known mass, the time to impact for each unknown analyte is converted into a *m/z* value.

For bacteria, the generated mass spectrum can be thought of as a unique protein profile. MALDI-TOF-MS will detect the most abundant proteins over a predefined mass range (typically 2 to 20 kDa). These are mostly intracellular, hydrophilic proteins and are primarily ribosomal proteins (Cherkaoui et al., 2010). Identification of the unknown bacteria is achieved by computerized comparison of the acquired spectra to a database of reference spectra composed of previously well-characterized bacterial isolates (Eigner et al., 2009).

3.3 Antibiotic resistance in bacteria indicative of faecal contamination

3.3.1 Antibiotics and antibiotic action on bacteria

Antibiotics are a family of pharmaceuticals used in the treatment of infectious diseases caused by microorganisms (Marti et al., 2014b). There are naturally produced antibiotics by microorganism (e.g. penicillin from the soil borne fungus *Penicillium*), antibiotics that are chemically synthesized and hybrid substances in which a naturally produced antibiotic is modified (semi-synthetic).

When a host is infected, bacteria can grow and multiply damaging the host. Antibiotics act on an important microbial structure or function of the bacterial cell interfering with an important cell process essential for growth and division to inhibit or destroy the bacterial population (Hooper, 2001). They can be divided into two classes based on their mechanism of action: bactericidal and bacteriostatic. Bactericidal antibiotics kill bacteria whilst bacteriostatic antibiotics slow their growth or reproduction. Antibiotics are usually classified

based on their structure or function. Table 3-3 summarises the antibiotic classes and their respective modes of action.

3.3.1.1 Inhibition of the bacterial cell wall synthesis

Antibiotics that interfere with the synthesis of the cell wall weaken the peptidoglycan scaffold within the bacterial wall so that the structural integrity eventually fails. Bacterial cells have a high osmotic internal pressure and without the structural cell wall will burst when subjected to hypotonic environments. The basic cell wall structure is a chain of disaccharide residues cross linked with peptide bridges creating a rigid mesh structure for the bacteria. The enzymes involved in the building of the cell wall chains are a target for antibiotics (e.g. cefpodoxime, amoxicillin and vancomycin). The binding of the antibiotic to the enzymatic target inhibits the assembly of the peptidoglycan chains. These enzymes are sometimes referred to as penicillin binding proteins as they are a target for β lactam antibiotics (Bugg et al., 2011). Vancomycin interacts with the D-alanine terminal of pentapeptide chains sterically interfering with the formation of the cross linking bridges (Watanakunakorn, 1984). Vancomycin is too large a molecule to pass through the outer membrane pores in Gram negative bacteria to reach the target peptidoglycan site. Disruption of the bacterial cell membrane function

The bacterial cytoplasmic cell membrane separates the cell from its environment and consists of phospholipids and proteins that regulate the movement of ions, nutrients and water in and out of the cell. Polypeptide antibiotics can distort the cell membrane by binding to the phospholipids in the membrane making them more permeable. This disrupts

the osmotic balance causing the leakage of cellular molecules, inhibits respiration and increases the uptake of water leading to cell death (Black, 1996).

Table 3-3: Antibiotic classes and their modes of action (from Black, 1996)

Class	Group	Example	Mode of action	Bacteriostatic /bactericidal
β Lactams	Penicillins Carbapenhams Cephalosporins	Amoxicillin Doripenem Cefpodoxime	Inhibition of cell wall synthesis	bactericidal
Macrolides		Clarithromycin	Inhibit protein synthesis	bacteriostatic
Tetracyclines		Oxytetracycline	Inhibit protein synthesis	bacteriostatic
Quinolones		Ciprofloxacin	Inhibits nucleic acid synthesis	bactericidal
Glycopeptides		Vancomycin	Interferes with cell wall synthesis	bacteriostatic
Aminoglycosides		Gentamycin	Inhibit protein synthesis	bacteriostatic
Sulfonamides		Sulfamethoxazole	Inhibit metabolic pathways.	bacteriostatic

3.3.1.2 Inhibition of nucleic acid synthesis

A nucleic acid inhibitor is a type of antibiotic that acts by inhibiting the production of nucleic acids and there are two major classes: DNA inhibitors and RNA inhibitors. Rifamycins inhibit RNA transcription. Quinolones (e.g. ciprofloxacin) are a key group of antibiotics that interfere with DNA synthesis by inhibiting the enzymes topoisomerase II (DNA gyrase) and

topoisomerase IV required for DNA replication, transcription, repair, and recombination (Marti et al., 2014a; Robicsek et al., 2006). Quinolones enter the cell through porins in the outer membrane and complex selectively and reversibly with DNA gyrase and topoisomerase IV resulting in the inhibition of supercoiling DNA. The DNA gyrase subunit is the primary quinolone target in gram negative bacteria, whereas topoisomerase IV is the primary target in Gram positive bacteria (Hooper, 2001).

3.3.1.3 Inhibition of protein synthesis

Macrolide antibiotics (e.g. clarithromycin) target the 23S ribosomal RNA (rRNA) of the 50S ribosomal sub-unit, which inhibits the formation of polypeptides. During translation, these antibiotics block the elongation step or peptide release step of protein synthesis. In Gram-negative bacteria there is limited entry into the cell because macrolides are lipophilic molecules and they are too large to pass through the aqueous porins of the cell membrane and therefore most Gram negative bacteria are resistant to macrolides (Retsema et al., 2001).

3.3.1.4 Inhibition of essential metabolites

Antibiotics in this group interfere with metabolic processes within the bacterial cell by mimicking or imitating the usual molecule required for the specific metabolic processes. Examples include sulfonilamide and trimethroprim (Black, 1996).

3.3.2 Mechanisms of antibiotic resistance

Antibiotic resistance describes the ability of bacteria to resist the action of antibiotic drugs. Some bacteria are naturally resistant to particular antibiotic agents; however, it is of great

concern to public health when bacteria that are normally susceptible to a particular agent become resistant. Bacteria have developed different mechanisms to resist antibiotics and the typical mechanisms that have been employed are summarised in Table 3-4.

3.3.3 Dissemination of antibiotic resistance

Bacteria generally become resistant to antibiotics due to changes in the bacterial genes either through mutations in the chromosomes, which are then inherited by their progeny (vertical transfer), or by the acquisition of extra-chromosomal DNA (e.g. plasmids) by horizontal gene transfer (Schwartz et al., 2003). Resistance due to changes in the chromosomes usually results in resistance to a single antibiotic group. However, bacteria can become resistant to a number of separate antibiotics by the horizontal gene transfer of extra-chromosomal DNA (Zhang et al., 2009).

Horizontal gene transfer generally can occur via three routes:

- **transformation:** the mechanism by which the cell can take up isolated DNA molecules from the medium surrounding it; this only happens at a certain stage of the growth cycle of the cell and is facilitated by competence factors (proteins). Not all bacteria can take up DNA molecules in this way (Droge et al., 1999).
- **transduction:** this mechanism involves the transfer of isolated DNA molecules by bacteria viruses (phages) and requires that both donor and recipient cells have surface receptors for phage binding and is therefore usually limited to closely related bacteria (Droge et al., 1999).
- **conjugation:** this mechanism involves the direct transfer of DNA through cell to cell contact and in most cases involves the transfer of plasmid DNA (Droge et al., 1999).

Table 3-4: Mechanisms of bacterial resistance to antibiotics (Black, 1996)

Mechanism	Description	Example antibiotics
Target alteration	Mutations in the DNA alter the antibiotic target site. Without binding, no inhibition is exerted	Macrolides Glycopeptides
Membrane permeability alteration	Membrane proteins that allow antibiotics into the cell change to prevent them entering the cell	Tetracyclines Quinolones Aminoglycosides
Enzymes	DNA encoding for enzymes that destroy or break down antibiotic active	β lactams
Metabolic pathway alteration	Genetic changes to bypass a metabolic pathway that the antibiotic exerts its effect on	Sulfonamides

Resistant genes can be transferred on extra chromosomal elements such as plasmids, transposons or integrons, all of which are thought to have played a major role in the spread of antibiotic resistance (Koczura et al., 2012; Henriques et al., 2006). Examples of the different types of mobile genetic elements associated with antibiotic resistance are given in Table 3-5. Plasmids are circular extra chromosomal double stranded DNA elements that can supplement the chromosomal DNA. They add an important extra dimension to the flexibility of the microorganism response to changes in its surrounding environment regardless of whether these changes are hostile (e.g. the presence of antibiotics or toxic materials) or favourable (e.g. the availability of a new substrate). Plasmid transfer can occur between bacteria of the same species or different species and even between closely related genera of bacteria (Schwartz et al., 2003). Replication of a plasmid within a bacterial cell depends on

the plasmid. Some plasmids can replicate independently of bacterial chromosome replication and other plasmids will only replicate when initiation of chromosomal replication occurs. Genes encoding for antibiotic resistance can move from one plasmid to another or become inserted into the bacterium chromosome on transposable elements. The transposition can occur irrespective of taxonomic class.

Table 3-5: Examples of mobile genetic elements found in bacteria for the transmission of antibiotic resistance. Taken from E. Marti et al. (2014)

Mobile genetic element	Characteristic	Examples
Plasmid	Self transmissible or mobilisable	pP2G1 contains ARGs
Insertion sequence (IS)	Encodes transposition	IS18 mediates overexpression of β-lactam ARG
Transposon (Tn)	Can be flanked by an IS and can encode for transposition and a functional gene e.g. ARG	Tn1 and Tn3 confer resistance to β-lactams
Integron	For the capture and expression of gene cassettes. Carries genes for integration and transcription	Class 1 contains gene cassettes conferring multidrug resistance
Genomic island	Mobile regions of DNA for encoding of complex functions	SGI1 confers resistance to streptomycin, β-lactams and sulphonamides
Integrating conjugative elements (ICE)	Transmissible mobile genetic elements that contain genes for conjugation and excision. Integrate and replicate in chromosome	ICEVchHai1 confers resistance to different antibiotics

ARG – antibiotic resistance gene

Transposable elements or transposons typically consist of genes required for transposition of one or more resistance genes and they can only replicate once inserted into the plasmid or chromosome. Transposons often contain mobile genetic elements that can capture genes situated in mobile gene cassettes called integrons (Koczura et al., 2012). Integrons consist of a promoter gene, integrase coding gene, recombination sites and resistance genes

(Henriques et al., 2006). They are genetic platforms that are responsible for the integration and rearrangement of gene cassettes and therefore resistance genes and consequently are considered a large contributor to the spread of multi resistance (Koczura et al., 2012). The acquisition of mobile genetic elements may cause a metabolic burden to the bacterial cell. However, studies conducted by Enne et al. (2005) and McDermott et al. (1993) have shown the acquisition of transposons conferring kanamycin resistance and ampicillin resistance can be a fitness advantage to *Escherichia coli*.

3.3.4 Antibiotic resistance in environmental waters

Antibiotic resistant bacteria have been detected in the aquatic environment (Birosova et al., 2014; Marti et al., 2014; Figueira et al., 2011; Zhang et al., 2009; Watkinson et al., 2007; Ash et al., 2002). The presence of resistant bacteria in surface waters can be attributed to a combination of the following factors: discharges of antibiotic residues and resistant bacteria with treated wastewater effluent, survival of resistant bacteria in surface waters and resistance transfer processes such as horizontal gene transfer. The presence of resistance elements in environmental waters and the presence of transferable elements within environmental bacteria support these conclusions (Amos et al., 2014; Marti et al., 2014; Kaplan et al., 2013; Schluter et al., 2007; Szczepanowski et al., 2007; Henriques et al., 2006; Pei et al., 2006; Tennstedt et al., 2005; Goni-Urriza et al., 2000).

A greater prevalence of resistant bacteria have been observed in wastewater treated effluents compared to influent wastewater, indicating wastewater treatment processes may contribute to the dissemination of antibiotic resistant bacteria (Silva et al., 2007; Silva et al., 2006). In addition, a correlation between resistant bacteria within river waters and urban

wastewater input has been reported (Leclercq et al., 2007), indicating that resistant bacteria can survive at sub inhibitory antibiotic concentrations. Furthermore, it has been reported that bacteria can transfer genetic elements (e.g. plasmids) whilst surviving in a wide range of environmental conditions such as low nutrients (Fernandez-Astorga et al., 1992).

3.3.5 Antibiotic resistance in *E.coli*

Escherichia coli and enterococci species (particularly *E.faecium* and *E.faecalis*) are frequently isolated from human infections (Health Protection Agency, 2007) and therefore it is important to monitor their resistance to the antibiotics used against them.

3.3.5.1 Resistance to β Lactams

Resistance to penicillins (e.g. amoxicillin) by *E.coli* is widespread and according to antibiotic resistance surveillance co-ordinated by the European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net) (2013), *E.coli* penicillin resistance in humans increased from 50.8% to 62.7% in the UK between 2000 to 2012. Over the same period, *E.coli* Resistance to 3rd generation cephalosporins (e.g. cefpodoxime) also increased (from 1.2 – 13.1 %).

In *E.*coli, resistance to penicillins usually occurs by the acquisition of plasmids carrying genes coding for β lactamases (Nafsika, 2007). β lactamases are enzymes that hydrolyse the β lactam ring of β lactam antibiotics rendering them inactive and more than 200 different β lactamases have been described. They can be classified by their amino acid sequence (Nafsika, 2007) and some are specific to penicillins, cephalosporins or carbapenems, whereas others have a broad range of activity. The nomenclature of β lactamases varies and

may refer to the patient they were first discovered in, the substrate, biochemical property, strain of bacteria or even location of the gene on the chromosome (Paterson et al., 2005). TEM type derivatives of β lactamases (named after the patient, Temoneria, this enzyme was first isolated from) are the most common type found in *E.coli* and account for up to 60% of penicillin *E.coli* resistance (European Antimicrobial Resistance Surveillance Network, 2011). Mutations in the basic amino acid structure of TEM or SHV β lactamases (named after the sulfhydryl substrate binding point in which the activity of the inhibition is considered variable) extend their spectrum of activity and enhance their hydrolysing ability conferring resistance to penicillins and cephalsporins (extended–spectrum β lactamases) (Paterson et al., 2005). Most extended spectrum β lactamases (ESBLs) can be inhibited by β lactamase inhibitors such as clavulanic acid.

3.3.5.2 Resistance to fluoroquinolones

When fluoroquinolones were first introduced for clinical use in the 1980s, the emergence of clinical resistance was considered negligible. However, fluoroquinolone resistance quickly emerged globally (Robicsek et al., 2006). Resistance to fluoroquinolones can arise through chromosomal mutations arising from stepwise mutations in the gene (*gyrA*, *parC*, and *parE*) coding for the sub-units of DNA gyrase and DNA topoisomerase IV (Nafsika, 2007). An accumulation of mutations results in an increase of minimum inhibitory concentration (Marians et al., 1997). Low level resistance to fluoroquinolones can occur through changes in outer membrane porins or by active efflux pumps (Nafsika, 2007). Since the late 1990s, plasmid mediated resistance has been identified (Kaplan et al., 2013) and can occur through the acquisition of Qnr proteins which inhibit the binding of fluoroquinolones (e.g.

ciprofloxacin) with DNA gyrase. There are several variations of these proteins that have been identified (*QnrA, QnrB, QnrC, QnrD and QnrS*) and acquisition of these genes can increase fluoroquinolone minimum inhibitory concentrations between 8 and 64 fold in *E.coli* (Martinez, 2009). Additionally, Qnr proteins have been identified in waterborne bacteria (Picao et al., 2008).

Resistance to fluoroquinolones is not widespread in human *E.coli* isolates. However, the European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net) (2013) reports an increase in fluoroquinolone resistant *E.coli* (6.2 % to 16.6 %) between 2000 and 2012.

3.3.6 Antibiotic resistance in enterococci

To assess the susceptibility of a species of enterococci, it is important to first identify the causative agent because resistance to some antibiotics can be intrinsic or more widespread in some species than others. Enterococci species are intrinsically resistant to a broad range of antibiotics including penicillins, cephalosporins (e.g. cefpodoxime), sulphonamides and aminoglycosides (European Antimicrobial Resistance Surveillance Network, 2011).

3.3.6.1 Resistance to β lactams

Intrinsically, enterococci have low level resistance to β lactamase antibiotics due to the low affinity penicillin binding proteins (e.g. PBP5) found on the cell wall. Loss of this non-essential protein renders strains highly susceptible with ampicillin minimum inhibitory concentration values (MICs) < 0.06 mg/L. Whereas, over expression of PBP5 has been correlated to ampicillin MIC values up to 64 mg/L (Eliopoulos, 2007). Target modifications in

the peptidoglycan cell wall chains have also been associated with ampicillin resistance in *E.faecium* (Mainardi et al., 2000). Contrary to *Escherichia coli*, the production of β lactamases is rare in enterococci. Resistance to penicillins is more widespread in *E.faecium* than in *E.faecalis*.

UK surveillance of penicillin resistance in *E.faecium* has indicated that resistant rates of human *E.faecium* isolates have increased from 77.6 % to 93.1 % during 2005 -2012 (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2014).

3.3.6.2 Resistance to vancomycin

The surveillance of vancomycin resistance in enterococci species is mandatory in clinical settings due to the importance of vancomycin therapy for enterococci infections. Vancomycin resistance occurs by the synthesis of modified cell wall precursors that express a decreased affinity for vancomycin and other glycopeptides. Low level resistance to vancomycin with susceptibility for teicoplanin, results from the presence of *vanC* type resistance determinants which are intrinsic to some species of enterococci (*E.gallinarum, E.casseliflavus and E.flavescens*) (Eliopoulos, 2007). These species of enterococci with intrinsic *vanC* resistance can acquire other *van* resistance genes (e.g. *vanA*, *vanB*, *vanC* and *vanD*) thereby increasing their resistance level. The *vanA* and *vanB* determinants confer high levels of vancomycin resistance which may be transferred by plasmids (European Antimicrobial Resistance Surveillance Network, 2011). Bacteria which are physiologically similar to enterococci, including Leuconostoc and Pediococcus species are also intrinsically resistant to high levels of vancomycin due to thickening of the cell wall and a decreased

affinity for vancomycin to cell wall precursors (Handwerger et al., 1994). Resistance to vancomycin in *E.faecalis* has remained less than 4% in the UK (2005 – 2010). In *E.faecium* resistance to vancomycin has decreased from 33.0 % to 13.3% from 2005 to 2010 in the UK (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2014b).

3.3.6.3 Resistance to macrolides

Macrolide antibiotics constitute an important alternative therapy for the treatment of insidious enterococci infections (Portillo et al., 2000) although, surveillance of macrolide (including clarithromycin) resistance in clinical isolates of *E.faecium* is not mandatory in England. Resistance to the macrolide antibiotics is common in enterococci bacteria, despite it not being intrinsic to the species (Eliopoulos, 2007). Gram negative bacteria are intrinsically resistant to macrolides due to the impermeability of the outer cell membrane. However, Gram positive bacteria can acquire resistance to macrolides by altering the ribosomal binding site through methylation (mediated by *erm* genes) or by efflux pumps (e.g. *mef* genes) that prevent the accumulation inside the cell (Pechere, 2001).

3.3.6.4 Resistance to fluoroquinolones

There is no mandatory surveillance of fluoroquinolone resistance in human *E.faecium* isolates in the UK, yet there are studies that report resistance to fluoroquinolones is widespread in the enterococci genus (Eliopoulos, 2007).

Resistance to fluoroquinolones can occur through mutations in the enzymes (topoisomerase) important for DNA replication and transcription or by efflux pumps

preventing the accumulation of the antibiotic inside the cell. Similarly to *E.coli*, the mutations are chromosomal arising from stepwise mutations in the genes (*gyrA* and *parC*) coding for the sub-units of DNA gyrase and DNA topoisomerase IV. An accumulation of mutations results in an increase of minimum inhibitory concentration.

3.4 Antibiotic susceptibility testing

Antibiotic susceptibility tests (AST) are carried out to determine which antibiotic will be successful in the treatment of a bacterial infection. There are generally two different AST methods that are used in clinical environments; the disc diffusion method and the agar dilution method (Huang et al., 2012). The disc diffusion method involves swabbing a uniform culture of the organism of interest on an agar plate and applying filter paper discs impregnated with a specific concentration of the antibiotic to be tested. The plates are then incubated and the antibiotic diffuses out from the filter paper, the concentration of the antibiotic being highest closer to the filter paper. Following incubation, any clear zones around the filter paper discs represent zones of the antibiotic inhibiting the growth of the organism (Black, 1996). Different antibiotics will diffuse at different rates and therefore interpretation of zones should only be compared to standard measurements such as those previously established for the antibiotic by the British Society for Antimicrobial Chemotherapy (BSAC, 2011). The disc diffusion test is a qualitative test that correlates inhibition zones to clinical break points to determine if the organism under test is resistant (R), sensitive (S) or of intermediate sensitivity (I) to the antibiotic (BSAC, 2011).

Minimum inhibitory concentrations values (MICs) are defined as the lowest concentrations of an antibiotic that will inhibit visible growth of a microorganism after overnight incubation

(Andrews, 2011). MIC values can be determined by agar or broth dilution techniques according to the standards established by various authorities such as the Clinical and Laboratory Standards Institute (CLSI, 2012) and the British Society for Antimicrobial Chemotherapy (BSAC, 2011). MIC values can also be evaluated using antibiotic gradient strips. The use of antibiotic gradient strips is technically straightforward as tests are set up in the same way as the disc diffusion method. Their versatility and ease of use make this method an attractive alternative to conventional dilution tests (Brown et al., 1991). However, antibiotic gradient strips are a quantitative technique for the determination of MIC values. They are plastic strips that are impregnated with 15 pre-defined antibiotic concentrations along the length of one side of the strip. On application to an inoculated agar surface, the antibiotic is released to the agar from the strip, forming a defined concentration gradient around the strip. After incubation an ellipse shaped zone of no growth will form where the ellipse meets the strip, and the MIC can be read from the concentration markings on the strip (Turndge, 2005). It has been reported that results from antibiotic gradient strips are as reliable as those obtained by the standard antimicrobial susceptibility testing methods (Mushtag et al., 2010; Hope et al., 2007; Hong et al., 1996).

There are different growth mediums available for antibiotic susceptibility testing including Mueller Hinton and Iso-Sensitest. Antibiotic Susceptibility protocols recommended by The European Committee on Antimicrobial Susceptibility Testing - EUCAST (2014) employ Mueller Hinton. The choice of the growth medium for antibiotic susceptibility testing is important as supplements or medium constituents may affect the growth of the organism and potentially the accuracy and reproducibility of the test Rennie et al. (2012)

Advances in molecular biological techniques can be used to detect antibiotic resistance and genotypic methods can also be used. DNA based assays have been developed for the detection of bacterial resistance genes. Genotypic methods do not provide information on antibiotic phenotypes but can identify resistance genes in bacteria that cannot be cultivated (Volkmann et al., 2004). The availability of molecular methods are not necessarily an improvement over phenotypic methods because the genetic mechanism responsible for the resistance needs to be known. If the mechanism is not known, no appropriate molecular assay can be developed. In addition, there may be more than one mechanism responsible which could lead to very complex assays. Finally if a resistance gene is detected it does not necessarily mean that it confers a resistance phenotype (Fluit, 2007).

3.4.1 Definition of resistance to antibiotics

Clinical breakpoint values are used in the clinical laboratory to advise on patient therapy. Breakpoints for phenotypic antimicrobial susceptibility testing have been determined by breakpoint committees (e.g. the British Society for Antimicrobial Chemotherapy, the European Committee on Antimicrobial Susceptibility testing and the Clinical and Laboratory Standards Institute) and as part of regulatory processes for the approval of new drugs. To determine breakpoint antibiotic doses, pharmakinetics, pharmacodynamics, resistance mechanisms, MIC value distributions and epidemiological cut-off values (ECOffs) are considered EUCAST (2010).

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has developed the concept of epidemiological cut-off values to aid the identification of the emergence of acquired resistance mechanisms (Kahlmeter et al., 2003). Epidemiological cut-off values

(ECOffs) are achieved by collecting MIC values from a population of bacteria from the same taxonomic group (genus or species). The MIC data is then pooled in a histogram and the ECOff values are then visually estimated or statistically calculated (Turnidge et al., 2006). An example of ciprofloxacin MIC values collected by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) for *E.coli* and the corresponding ECOff value is shown in Figure 3-1. ECOff values are used to distinguish between bacteria with acquired resistance mechanisms (non-wild type) and bacteria without acquired resistance mechanisms (wild type) (Kahlmeter et al., 2003).

The EUCAST breakpoint definitions (EUCAST, 2012a) are as follows:

- clinically Susceptible (S) - The microorganism is defined as susceptible if the antibiotic will have therapeutic success at a particular concentration.
- clinically Resistant (R) **–** If an applied antibiotic concentration has a high likelihood of therapeutic failure then the microorganism is considered resistant.
- Wild type (WT) - a microorganism is defined as WT by the absence of acquired and mutational resistance mechanisms to an antibiotic. A microorganism is categorized as WT for a species by applying the appropriate cut-off value in a defined phenotypic test system. Wild type microorganisms may or may not respond clinically to antimicrobial treatment.
- Non Wild type (NWT) - a microorganism is defined as non-wild type by the presence of an acquired or mutational resistance mechanism to an antibiotic. A microorganism is categorized NWT for a species by applying the appropriate cut-off

value in a defined phenotypic test system. NWT microorganisms may or may not respond clinically to antimicrobial treatment.

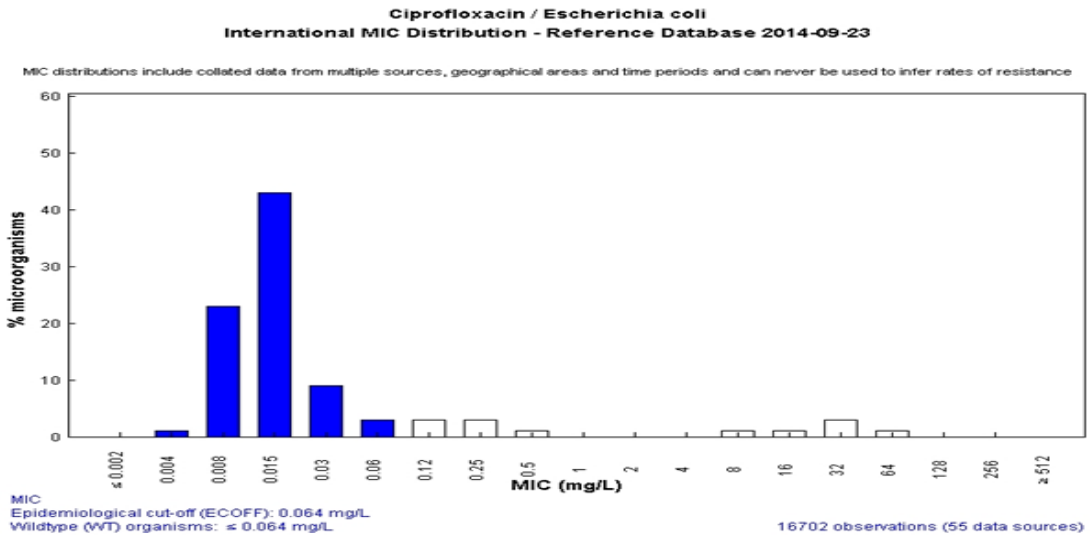


Image source: the European Committee of Antimicrobial Susceptibility Testing.

Blue and white bars represent wild and non-wild type respectively (EUCAST, 2014)

Figure 3-1: Histogram showing the distribution of ciprofloxacin minimum inhibitory concentration (MIC) values measured for *Escherichia coli* isolates submitted to the European Committee of Antimicrobial Susceptibility Testing (EUCAST).

4 Detection of Pharmaceuticals in the Urban Water Environment

4.1 Introduction

Due to advancing analytical techniques, there are increasing numbers of publications reporting the detection of trace levels of pharmaceuticals in wastewater treatment plant influents (Zorita et al., 2009; Karthikeyan et al., 2006), effluents (Stülten et al., 2008; Brown et al., 2006; Clara et al., 2005) and in river waters (Gros et al., 2007; Gros et al., 2006; Moldovan, 2006; Vieno et al., 2006). Collectively, researchers have demonstrated that few pharmaceuticals are completely removed during wastewater treatment and can therefore be discharged to receiving waters. However, the concentrations reported vary due to differences in the types of wastewater treatment employed, population size and critically, the prescription quantities.

In this chapter, an analytical method to detect a selection of pharmaceuticals in environmental waters is described and used to investigate the passage of the selected pharmaceuticals through a large urban wastewater treatment plant (WWTP) employing activated sludge. The concentrations of target pharmaceuticals in wastewater samples collected from different stages of the wastewater treatment process are compared and the reductions of these compounds estimated. The impact of the treated effluent on the receiving surface water is assessed by comparing the pollutant concentrations up-stream and down-stream of the discharged effluent.

4.1.1 Selection of pharmaceuticals

The pharmaceuticals originally selected for monitoring throughout the wastewater treatment process were amoxicillin, bezafibrate, carbamazepine, ciprofloxacin, and clarithromycin. These compounds were selected as they have different physicochemical properties (presented in Section 4.3.2) and therefore provide the opportunity to investigate the pathways of chemically different compounds through the wastewater treatment process. Selection was also based on prescription quantities estimated for the WWTP catchment (given in Section 4.3.5) and the detection in environmental waters reported by other studies. Finally, the success of the analytical method to determine these compounds in environmental waters was important in the compound selection (Section 4.3.2).

Additional antibiotics, vancomycin and cefpodoxime, were selected for the work described in Chapter 5 due to their importance in the treatment of nosocomial infections and because they are considered critically important to human medicine (see Chapter 5, Section 5.1.2). However, the analytical methodology described in this chapter did not include these compounds due to the very low prescription levels (0.025 and 0.002 Tonnes a^{-1} for vancomycin and cefpodoxime respectively) for the community in England leading to very low environmental concentrations (see Table 4-2 and Table 4-3). All the compounds selected for the studies described in Chapters 4-6 are only available on prescription in England and their structures and International Union of Pure and Applied Chemistry (IUPAC) names are given in Table 4-1.

Table 4-1: Structure and properties of the pharmaceuticals selected for this study

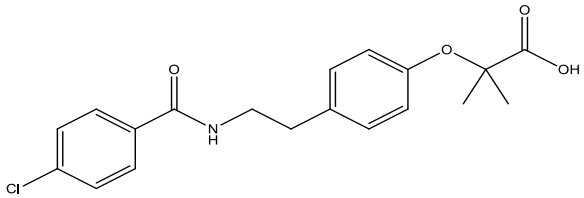
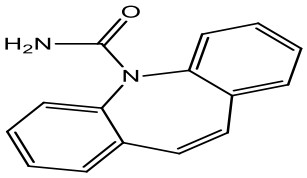
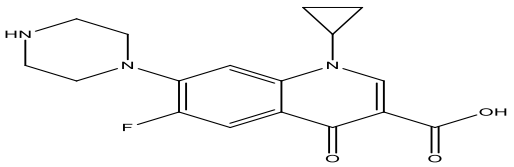
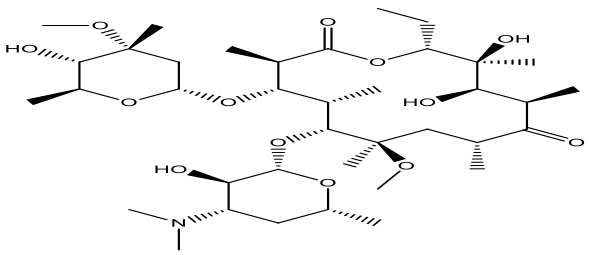
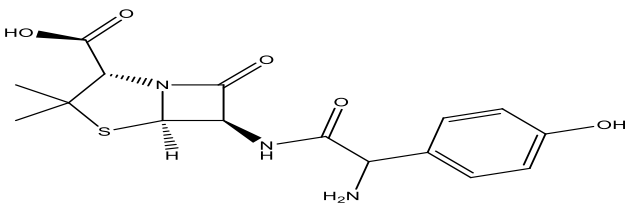
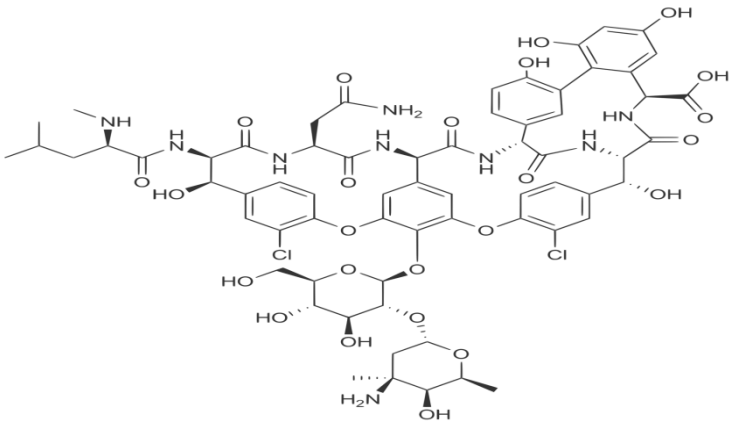
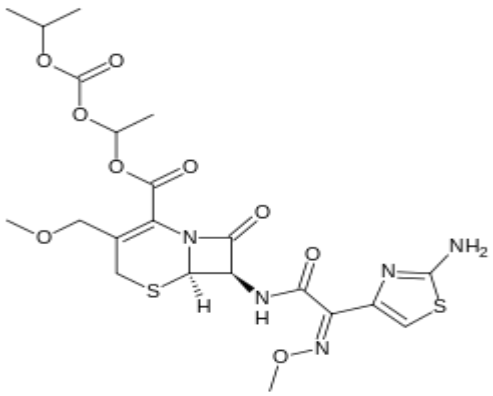
a. Pharmaceutical b. Class c. CAS number d. Formula e. MW (g/mol)	Structure and IUPAC name	i. pKa ii. Sol (mg/L) iii. Log KOW iv. KOC
a. Bezafibrate b. Lipid regulator c. 41859-67-0 d. $C_{19}H_{20}ClNO_4$ e. 361.8	 2-(4-{2-[(4-chlorobenzoyl)amino]ethyl}phenoxy)-2-methylpropanoic acid	i. 3.6[a] ii. 1.5[c] iii. 4.25[a] iv. 2.5[a]
a. Carbamazepine b. Anticonvulsant c. 298-46-4 d. $C_{15}H_{12}N_2O$ e. 236.3	 5H-dibenzo [b,f]azepine-5-carboxamide	i. 13.9[a] ii. 18.0[b] iii. 2.45[a] iv. 510[b]
a. Ciprofloxacin b. Quinolone antibiotic c. 85721-33-1 d. $C_{17}H_{18}FN_3O_3$ e. 331.3	 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-quinoline-3-carboxylic acid	i. 6.1, 8.7[b] ii. 30,000[b] iii. -0.4[a] iv. 61,000[b]
a. Clarithromycin b. Macrolide antibiotic c. 81103-11-9 d. $C_{38}H_{69}NO_{13}$ e. 747.9	 3R,4S,5S,6R,7R,9R,11S,12R,13S,14S)-6-{[(2S,3R,4S,6R) -4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy} -14-ethyl-12,13-dihydroxy-4-{[(2R,4S,5S,6S)-5-hydroxy -4-methoxy-4,6-dimethyloxan-2-yl]oxy}-7 -methoxy-3,5,7,9,11,13-hexamethyl -1-oxacyclotetradecane-2,10-dione	i. 8.99[a] ii. 0.3[c] iii. 3.16[a] iv. 150[b]
a. Amoxicillin b. B Lactam antibiotic c. 26787-78-0 d. $C_{16}H_{19}N_3O_5S{\cdot}3H_2O$ e. 365. 4	 (2S,5R,6R)- 6-{[(2R)-2-amino- 2-(4-hydroxyphenyl)- acetyl]amino}- 3,3-dimethyl- 7-oxo- 4-thia- 1-azabicyclo[3.2.0]heptane- 2-carboxylic acid	i. 2.4,7.4,9.6[a] ii. 3740[c] iii. 0.87[a] iv. N/A

Table 4-1 (continued)

Pharmaceutical CAS number Formula MW (g/mol)	Structure	i. pKa ii Sol (mg/L) iii Log KOW iv KOC
a. Vancomycin b. Glycopeptide antibiotic c. 1404-90-6 d. $C_{66}H_{75}Cl_2N_9O_{24}$ e. 1449.3	 (1S,2R,18R,19R,22S,25R,28R,40S)-48-{[(2S,3R,4S,5S,6R)-3-{[(2S,4S,5S,6S)-4-amino-5-hydroxy-4,6-dimethyloxan-2-yl]oxy}-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-22-(carbamoylmethyl)-5,47-dichloro-2,18,32,35,37-pentahydroxy-19-[(2R)-4-methyl-2-(methylamino)pentanamido]-20,23,26,42,44-pentaoxo-7,13-dioxa-21,24,27,41,43-pentaazaoctacyclo[26.14.2.2^{3,6}.2^{14,17}.1^{8,12}.1^{29,33}.0^{10,25}.0^{34,39}]pentaconta-3,5,8,10,12(48),14,16,29(45),30,32,34,36,38,46,49-pentadecaene-40-carboxylic acid	i. 2.6,7.2,8.6[c] ii. 225[c] iii. 1.11[c] iv. N/A
a. Cefpodoxime b. Cephalosporin antibiotic c. 82619-04-3 d. $C_{15}H_{17}N_5O_6S_2$ e. 427.5	 (6R,7R)-7-[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-(methoxyimino)acetamido]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid	i. 3.22[c] ii. 185[c] iii. 0.05[c] iv. N/A

MW - Molecular weight, IUPAC - The International Union of Pure and Applied Chemistry, pKa = acid dissociation constant, Sol =solubility, KOC = Soil organic partition coefficient, KOW = octanol water coefficient, N/A = data not available, [a] Ternes et al. (2008), [b] Wexler Philip (2001), [c] Wishart et al. (2008).

Bezafibrate is an amphipathic carboxylic acid compound belonging to a group of fibrate drugs that are used to regulate lipid levels in the blood. Approximately 50 % of the drug remains un-metabolised and is excreted as the parent compound (Garcia-Ac et al., 2009). Bezafibrate has been detected in environmental waters including wastewaters and surface waters at the levels reported in Table 4-2 and Table 4-3. However, results of toxicology tests (Isidori et al., 2007) indicate that there are no toxic adverse effects in non-target organisms at the fibrate concentrations (including bezafibrate) typically found in the environment.

Carbamazepine is an anticonvulsant used to treat epilepsy and is also used as an antidepressant. It is a neutral compound, moderately soluble in water and contains both a hydrophobic moiety (two phenyl functional groups) and a hydrophilic urea moiety. Carbamazepine is heavily metabolized in the human body. More than 30 metabolites have been identified in humans including 10, 11 –dihydro- 10, 11 epoxycarbamazepine and 10, 11 –dihydro- 10, 11 dihydroxycarbamazepine (Bahlmann et al., 2014) which are excreted via urine and faeces. Although, only a low percentage (approximately 10 %) is excreted as the unchanged compound, carbamazepine has been frequently detected in environmental waters (see Tables 4-2 and 4-3). No adverse acute toxicity effects of carbamazepine to juvenile rainbow trout were observed at environmental relevant concentrations (1.0 μg/L) by Li et al. (2011). In addition, Zhang et al. (2012) found carbamazepine did not exhibit an acute toxicity effect on algae. However, more chronic toxicity tests should be performed to gain more information of the effects of this compound and other pharmaceuticals in the environment.

Table 4-2: Reported concentrations of selected pharmaceuticals in wastewaters.

Compound	Sample	Concentration (ng/L)	Location	Treatment employed	Reference
Bezafibrate	Influent	209 -1391	Wales	2TBF (111,000)	Kasprzyk-Horden et el
	Effluent	< 85 – 665			(2009a)
	Influent	1960-7600	Austria	Various 2B	Clara et al. (2005)
	Effluent	nd-4800			
	Effluent	100 -510	Spain	2CAS (140,000)	Pedrouzo et al. (2011)
	Influent	200-460	Spain	Various 2CAS	Gracia-Lor et al. (2012)
	Effluent	20-390		(18,000-265,000)	
	Influent	135 – 1285	Wales	2CAS (30,000)	Kasprzyk-Horden et el
	Effluent	<94-393			(2009b)
	Influent	5.7 – 945.9	Greece	Various 2CAS (20,000-100,000)	Kosma et al. (2014)
	Influent	971[a]	Wales	2TBF (111,000)	Kasprzyk-Hordern et al.
	Effluent	418[a]			(2008b)
	Influent	420 ± 300	Finland	Various 3B (3000-740,000)	Lindqvist et al. (2005)
	Influent	121 ±108	Spain	3B (20,000)	Collado et al. (2014)
	PST	1900-2980	Spain	Various 2B (277, 000)	Radjenovic et al. (2009)
Carbamazepine	Influent	2 - 405	Spain	2CAS (140,000)	Pedrouzo et al. (2011)
	Effluent	8 - 170			
	Influent	290-400	Finland	Various 3B	Vieno et al. (2006)
	Effluent	380-470			
	Influent	570 ± 390	Italy	2CAS (120,000)	Verlicchi et al. (2014)
	Effluent	370 ± 69			
	Effluent	240[a]	Italy	2CAS (138000)	Al Aukidy et al. (2012)
	Influent	325 – 1850	Austria	2CAS (7000)	Clara et al. (2005)
	Effluent	465 - 1594			
	Influent	28.0 - 416.8	Greece	Various 2CAS	Kosma et al. (2014)
	Influent	709 -2930	Wales	2TBF (111,000)	Kasprzyk-Horden et el
	Effluent	104 - 3110			(2009a)
	Influent	27 ± 24	Spain	3B (20,000)	Collado et al. (2014)
	Influent	644 - 4596	Wales	2CAS (30,000)	Kasprzyk-Horden et el
	Effluent	152 - 2324			(2009b)

Table 4.2 (continued)

Compound	Sample	Concentration (ng/L)	Location	Treatment employed	Reference
Ciprofloxacin	PST	84-721	Canada	NA	Lee et al. (2007)
	Effluent	42-392			
	Influent	200-650	Finland	Various 3B	Vieno et al. (2006)
	Effluent	<29-40			
	Influent	1100[a]	Australia	Various	Watkinson et al. (2009)
	Effluent	ND			
	Influent	200-650	Finland	Various 2CAS	Vieno et al. (2007)
	Effluent	<29-40		(12,400-40,000)	
	Influent	2200 ± 1800	Italy	2CAS (120,000)	Verlicchi et al. (2014)
	Effluent	630 ± 349			
	Influent	313-568	Switzerland	NA	Golet et al. (2002)
	Effluent	62-106			
	Influent	513	Italy	2CAS (229,000)	Zuccato et al. (2010)
	Effluent	147			
	Influent	392 ± 218	Spain	3B (20,000)	Collado et al. (2014)
	PST	6900[a]	Australia	2CAS (700,000)	Watkinson et al. (2007)
	Effluent	720[a]			
	Influent	143-1304	Croatia	Various	Senta et al. (2008)
	Effluent	46-427			
Clarithromycin	Influent	112	USA	3B	Loganathan et al.
	Effluent	ND			(2009)
	Influent	200 ± 320	Italy	2CAS (120,000)	Verlicchi et al. (2014)
	Effluent	280 ± 24			
	Influent	138-1396	Croatia	Various	Senta et al. (2008)
	Effluent	103-996			
	Influent	<LOQ-724.2	USA	NA	Spongberg et al. (2008)
	Effluent	<LOQ-610.0			
Amoxicillin	Influent	280[a]	Australia	2CAS (700,000)	Watkinson et al. (2007)
	PST	270[a]			
	Influent	18	Italy	2CAS (229,000)	Zuccato et al. (2010)
	Effluent	ND			
Vancomycin	Influent	41	Italy	2CAS (229,000)	Zuccato et al. (2010)
	Effluent	40			

[a] Maximum detected. Population equivalent given in parenthesis if available, NA = Data not available, LOQ = limit of quantification, 3B =biological with tertiary treatment, 2CAS = secondary conventional activated sludge treatment, TBF = trickling bed filter, 2B = secondary biological treatment. PST = effluent from primary sedimentation tank. ND = not detected.

Table 4-3: Reported concentrations of selected pharmaceuticals in surface waters.

Compound	Sample matrix	Concentration (ng/L)	Location	Reference
Bezafibrate	Down-stream	< 50–130	Germany	Wiegel et al. (2004)
	Down-stream	58	Wales	Kasprzyk-Hordern et al. (2008a)
	Up-stream	41		
	Down-stream	4.5[a]	Sweden	Lindqvist et al. (2005)
	Up-stream	4.0[a]		
	Down-stream	25.5[a]	Spain	Silva et al. (2011)
	Down-stream	76[a]	Wales	Kasprzyk-Hordern et al. (2008b)
	Various	1.6	Serbia	Petrovic et al. (2014)
	Down-stream	200[a]	Canada	Metcalfe et al. (2003)
	Various surface water	3.4[b]	France	Vulliet et al. (2011)
	Various surface water	0.3-46	Spain	Fernan¡ndez et al. (2010)
	Down-stream	63	Spain	Garcia-Ac et al. (2009a)
	Down-stream	90[a]	Wales	Kasprzyk-Hordern et el (2009b)
	Up-stream	66[a]		
Carbamazepine	Down-stream	13.5[a]	US	Spongberg et al. (2008)
	Up-stream	ND		
	Down-stream	684[a]	Wales	Kasprzyk-Hordern et al. (2008b)
	Various	35.6	Serbia	Petrovic et al. (2014)
	Down-stream	4.0[a]	US	Conley et al. (2008)
	Up-stream	5.6[a]		
	Various surface water	2.9-23.1	US	Conley et al. (2008b)
	Various surface water	6720[a]	France	Feitosa-Felizzola et al. (2009)
	Various surface water	30 -250	Switzerland	Öllers et al. (2001)
	Down-stream	650[a]	Canada	Metcalfe et al. 2003
	Down-stream	6-11	Canada	Garcia-Ac et al. (2009b)
	Various surface water	13.9[b]	France	Vulliet et al. (2011)
	Various surface water	0.3 -104	Spain	Fernan¡ndez et al. (2010)
	Down-stream	53.8[a]	Spain	Silva et al. (2011)
	Down-stream	495[a]	Wales	Kasprzyk-Hordern et el (2009b)
	Up-stream	647[a]		
	Down-stream	112-67715	Spain	Valcarcel et al. (2011)
	Down-stream	< 30 – 140	Germany	Wiegel et al. (2004)

Table 4-3 (continued)

Compound	Sample matrix	Concentration (ng/L)	Location	Reference
Ciprofloxacin	Down-stream	< LOD (24)-25	Finland	Vieno et al. (2007)
	Up-stream	< LOD (24)		
	Down-stream	135 ± 2	France	Tuc Dinh et al. (2011)
	Various	28.2[a]	Serbia	Petrovic et al. 2014
	Down-stream	9-280	USA	Batt et al. (2006)
	Down-stream	130[a]	Spain	Gros et al. (2006)
	Up-stream	ND		
	Various surface water	4.7 -54.2	USA	Conley et al. (2008b)
	Down-stream	< 4.5	USA	Conley et al. (2008)
	Various surface water	26.2[a]	Italy	Castiglioni et al. (2004)
	Down-stream	<6-224	Spain	Valcarcel et al. (2011)
	Various surface water	9660[a]	France	Feitosa-Felizzola et al. (2009)
	Down-stream	1300[a]	Australia	Watkinson et al. (2009)
	Down-stream	1.32 – 26.89	Italy	Zuccato et al. (2010)
Clarithromycin	Down-stream	4.6 – 9.0	USA	Spongberg et al. (2008)
	Up-stream	1.4		
	Down-stream	36.9[a]	Spain	Silva et al. (2011)
	Various surface water	2330[a]	France	Feitosa-Felizzola et al. (2009)
	Various surface water	20.3[a]	Italy	Castiglioni et al. (2004)
	Down-stream	75	Switzerland	McArdell et al. (2003)
	Various surface water	616[a]	Serbia	Petrovic et al. (2014)
	Down-stream	130-1727	Spain	Valcarcel et al. (2011)
	Down-stream	250[a]	Spain	Gros et al. (2006)
	Up-stream	60[a]		
	Down-stream	0.89-36.0	Italy	Zuccato et al. (2010)
	Down-stream	< 30 - 40	Germany	Wiegel et al. (2004)
Amoxicillin	Down-stream	200[a]	Australia	Watkinson et al. (2009)
	Down-stream	68[c]	France	Tuc Dinh et al. (2011)
	Down-stream	622[ac]	Wales	Kasprzyk-Hordern et al. (2008b)
	Various surface water	ND	Italy	Castiglioni et al. (2004)
	Down-stream	3.57 – 9.91[c]	Italy	Zuccato et al. (2010)
Vancomycin	Down-stream	0.44 – 5.17	Italy	Zuccato et al. (2010)
	Down-stream	90	France	Tuc Dinh et al. (2011)

ND = not detected, [a] maximum detected, [b] Mean value detected, [c] Semi quantitative method used

Ciprofloxacin is a broad spectrum antibiotic used to treat bacterial infections. The structure of ciprofloxacin contains a carboxylic acid functional group which can be deprotonated and an amino group in the heterocyclic ring (piperazinyl) which can be protonated. Therefore the compound is amphoteric in nature and may exist in cationic, zwitterionic, or anionic forms depending on the pH conditions. Following ingestion, ciprofloxacin is partially metabolised in the liver but renal excretion is the primary route and approximately 50 % can be excreted as the original compound. Ciprofloxacin has been detected in environmental waters (see Tables 4-2 and 4-3) which is a concern as it's low biodegradability and toxic effects towards environmental bacteria have been well documented (Kümmerer et al., 2000, Hartmann et al., 1998). In addition, environmental risk assessments conducted by Halling-Sorensen et al. (2000) and Martins et al. (2012) identified ciprofloxacin as a potential risk to aquatic organisms.

Clarithromycin is a semi synthetic macrolide antibiotic derived from erythromycin and possesses hydrophobic properties. The structure of clarithromycin consists of a macro-cyclic 14-membered lactone ring attached to two sugar moieties (cladinose and desoamine). Clarithromycin is eliminated by urinary and biliary excretion and approximately 20 % can be excreted as the parent compound. There are reports of the detection of clarithromycin in environmental waters (see Tables 4-2 and 4-3). To assess the acute toxicity of clarithromycin towards aquatic organisms, toxicity tests were performed on bacteria, algae, rotifers, microcrustaceans and fish by Isidori et al. (2005). It was found that adverse effects did not occur due to clarithromycin at concentrations typically observed in the environment (ng/L).

Results from chronic toxicity tests indicated that clarithromycin is toxic to algae (EC50 range between 2.0 – 12000 μg/L depending on the species).

Both amoxicillin and cefpodoxime are classed as β lactam antibiotics due to the β lactam ring within their molecular structure which is responsible for their antibacterial activity. They differ in their spectrum of activity due to variations in the side chains within their molecular structure and belong to different antibiotic subgroups; penicillins and cephalosporins respectively. Amoxicillin is a widely used antibiotic (Andreozzi et al., 2004). The prescription levels in England are high (~ 163 Tonnes a^{-1} in 2012) and most of the ingested active ingredient is excreted unchanged (DrugBank, 2005). However, the prescription quantities of cefpodoxime are very low (0.002 Tonnes a^{-1} in England in 2012). Pharmacokinetic studies have demonstrated that over 80 % of a cefpodoxime dose is excreted unchanged in the urine (Tremblay et al., 1990) but a study of 200 surface and wastewater samples failed to detect a variety of β lactams (including penicillins and cephalosporins) (Cha et al., 2006). However, there are some studies that report the prevalence of β lactam antibiotics in sewage influents, settled sewage and surface waters (see Tables 4-2 and 4-3). Nevertheless, despite the high usage of β lactams, the number of reports is less frequent than for other antibiotics (Tables 4-2 and 4-3).

The rare detection of β lactams in environmental waters is possibly due to their low stability in aqueous solution. The β lactam ring is rapidly hydrolysed in aqueous solution, with maximum stability at pH 6.0-7.2. In addition, transition metals (e.g. mercury, zinc and copper) catalyse their degradation (Ternes et al., 2008). Due to the low prescription

quantities for cefpodoxime in England, this compound was not included for the work in this study. Toxicity tests have demonstrated that amoxicillin, present at concentrations between 50 ng/L and 50 mg/L is not toxic to algae species. However, hazard quotients estimated by Park et al. (2008) and acute toxicity tests reported by Wang et al. (2012) demonstrate that environmentally relevant concentrations of penicillins and cephalosporins pose a potential ecological risk.

Vancomycin is a glycopeptide antibiotic widely used in the US but in Europe it is reserved for the treatment of bacterial infections where other antibiotics have proven ineffective (Kümmerer, 2009). Following ingestion, more than 80 % of a dose of vancomycin is excreted in the original active form (Matzke et al., 1986). Despite the low consumption levels of vancomycin in Europe, its presence in wastewater and surface water has been reported in France and Italy at low concentrations (ng/l), although these reports are rare compared to the more frequent reports of other antibiotics (Table 4-2 and Table 4-3). The prescription quantities in England are very low (0.025 Tonnes a^{-1} in 2012) and hence this antibiotic was not selected for the analytical methodology work described in this chapter. Ecotoxicity data for vancomycin is limited, although high concentrations (1 – 6 mg/l) which are well above those detected in environmental waters, have been found to inhibit anaerobic sludge micro-organisms (Wexler Philip, 2009).

4.2 Materials and methods for the analysis of pharmaceuticals in environmental waters

4.2.1 Chemicals and reagents

Methanol, acetonitrile, formic acid, ammonium hydroxide, acetic acid and ammonium acetate were purchased from Fisher Scientific UK Ltd (Leicestershire, UK) and were either HPLC or LCMS grade. Reagent grade sulphuric acid and nitric acid were also purchased from Fisher Scientific UK Ltd. Ciprofloxacin (> 98 % HPLC), carbamazepine (>98 % HPLC, bezafibrate (> 98% HPLC) and clarithromycin (> 98 % HPLC) were purchased from Sigma-Aldrich Company Ltd (Dorset, UK)

4.2.2 Description of study area

The studied wastewater treatment plant (WWTP) is a large urban treatment plant in London, UK and is shown in Figure 4-1. It is the ninth largest WWTP in England and receives approximately 244,000 m^3 of wastewater per day from a 399 km^2 urban catchment serving a total population of approximately 870,000. The treatment plant applies primary sedimentation and secondary activated sludge treatment and has a hydraulic retention time of approximately 13 h (Ellis, 2006).

Sewage entering the plant is first screened for rags and grit. The screened sewage is then treated in one of sixteen primary settling tanks where solids are settled out and pumped to the sludge treatment plant for anaerobic digestion. The settled sewage is pumped to aerated activated sludge basins (twelve basins in total) followed by final settlement tanks

(thirty two tanks) in order to remove organic pollutants (Thames Water, 2012). A more detailed description of the WWTP process is given in Chapter 2, Section 2.2.1.2.

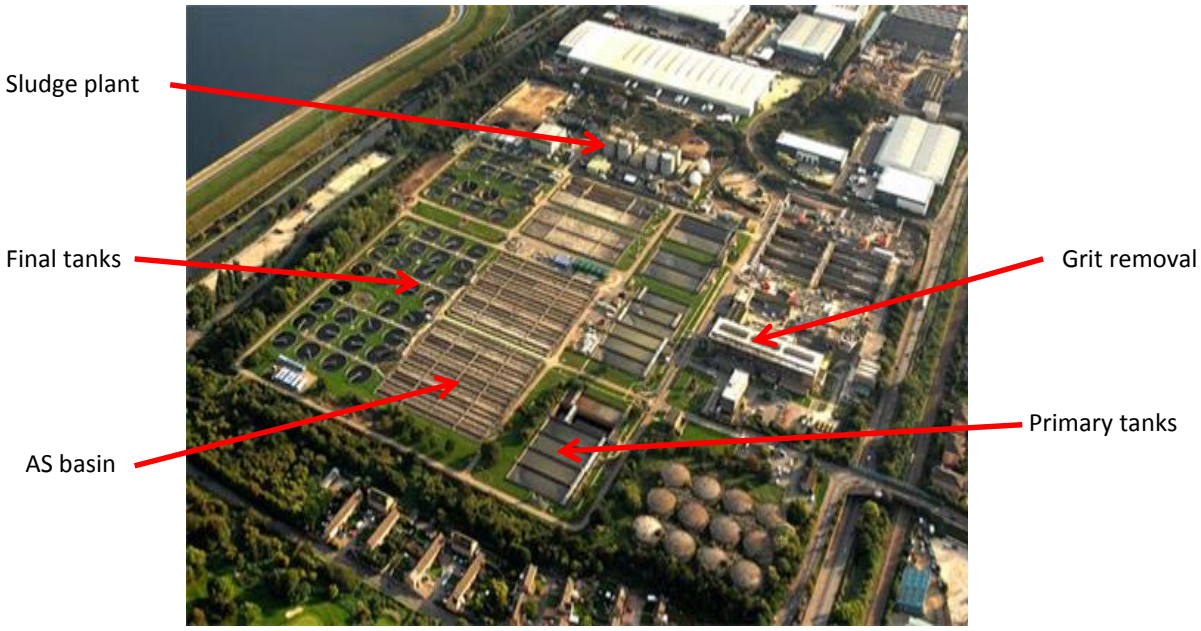


Image source: AECOM (2014)

Figure 4-1: Arial view of the WWTP from which samples were collected. Arrows identify the locations for grit removal, the primary settling tanks, the activated sludge basins (AS) and the final settlement tanks.

The treated effluent from the final settlement tanks is discharged through the final effluent channel into Pymmes Brook via Salmons Brook, both of which are tributaries of the River Lee. In addition, partially treated sewage maybe discharged to the Salmons Brook during periods of high rainfall. The River Lee is a large lowland river extending over 85 km from its source near Luton to its confluence (Bow creek) with the Thames. The river is divided into two catchments referred to as the upper and lower Lee. Agriculture is more dominant in the

upper Lee catchment compared to the lower Lee where the watercourse flows through predominantly urban areas (Snook et al., 2004).

The lower Lee river system has been heavily modified over the last century to cope with increasing urbanisation and to reduce the risk of flooding in the catchment. There are sections of the river in which the flow is split in to two or more parallel channels including a canalised channel (River Lee navigation) and the Flood Relief Channel (River Lee Diversion) (Davies, 2011). In addition, there are a number of tributaries including Nazeing Brook, Turnford Brook, Salmons Brook, Ching Brook and Pymmes Brook. The river system within the lower Lee catchment feeds a number of reservoirs collectively known as the Chingford reservoirs. Water abstracted from the River Lee accounts for approximately one-sixth of London's water supply (Snook et al.,2004)

The lower Lee has historically suffered from poor water quality. A substantial contribution to the pollution of the River Lee is due to discharges from sewage treatment works and in dry weather, the base flow down-stream of Pymmes Brook, is mainly treated effluent (The Environment Agency, 2013). In addition, widespread pollution within the River Lee has been attributed to sewer misconnections and combined sewer overflow discharges of sewage to surface water (Thames Water, 2014; The Environment Agency, 2013).

4.2.3 Sample collection

Wastewater samples were collected at three points representing different stages of the treatment process (see Figure 4-2).

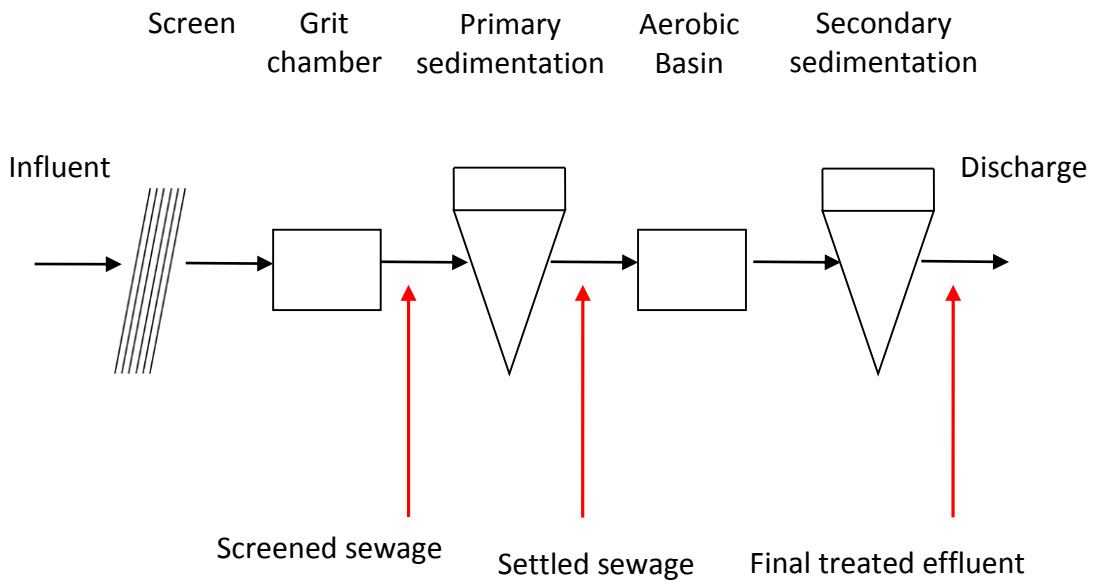
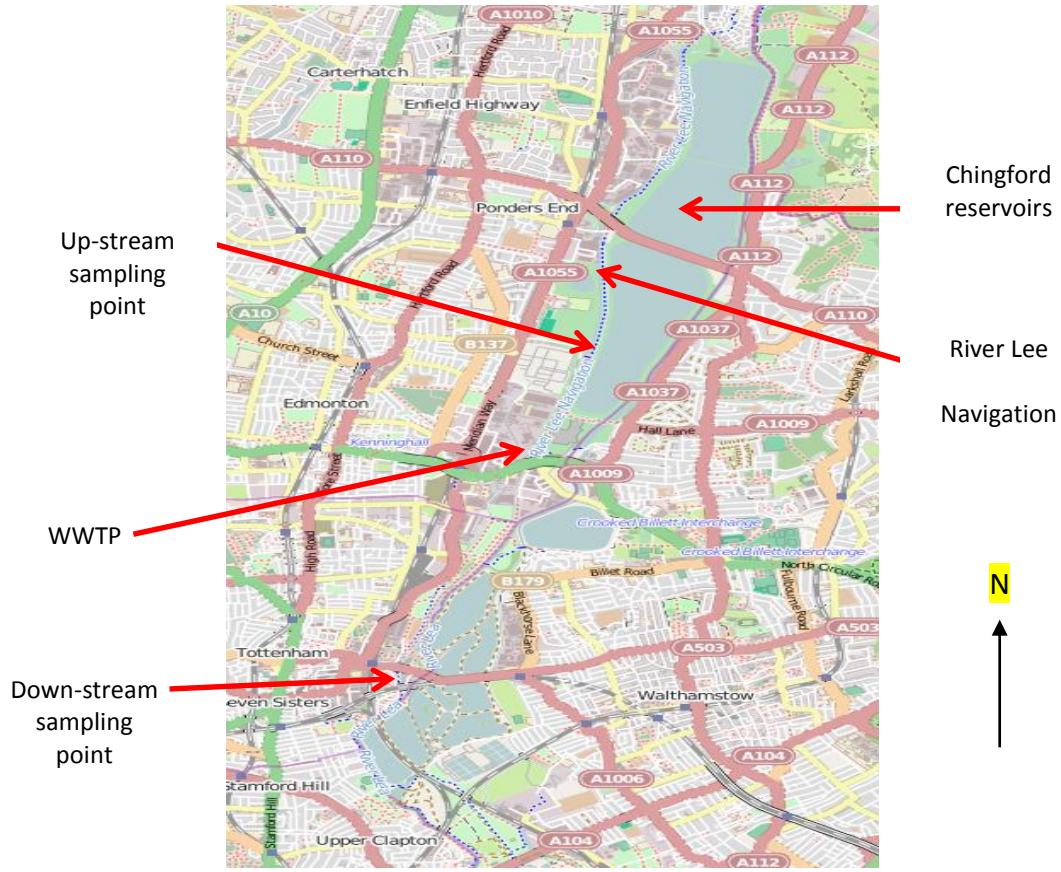


Figure 4-2: Schematic of the WWTP sampled in this study. Red arrows indicate the positions of sampling points.

In this study, screened sewage refers to sewage following gross solid and grit removal. Settled sewage refers to sewage that has passed through primary settlement tanks (the primary sedimentation treatment stage). Final treated effluent refers to sewage that has been treated by activated sludge followed by final sedimentation (in final settlement tanks). Whilst the plant remained operational, at the time of sampling the WWTP was undergoing an engineering upgrade to replace ageing infrastructure and to increase capacity in line with predicted population expansion.

Surface water samples were collected from the River Lee (Lee navigation channel) from positions both up- and down-stream of the Pymmes Brook confluence with the River Lee and therefore up- and down-stream of the WWTP treated effluent discharge point (Figure

4-3). Figure 4-4 shows in more detail the confluence of the Pymmes Brook with the River Lee and the location of the sampling point down-stream of the confluence point.

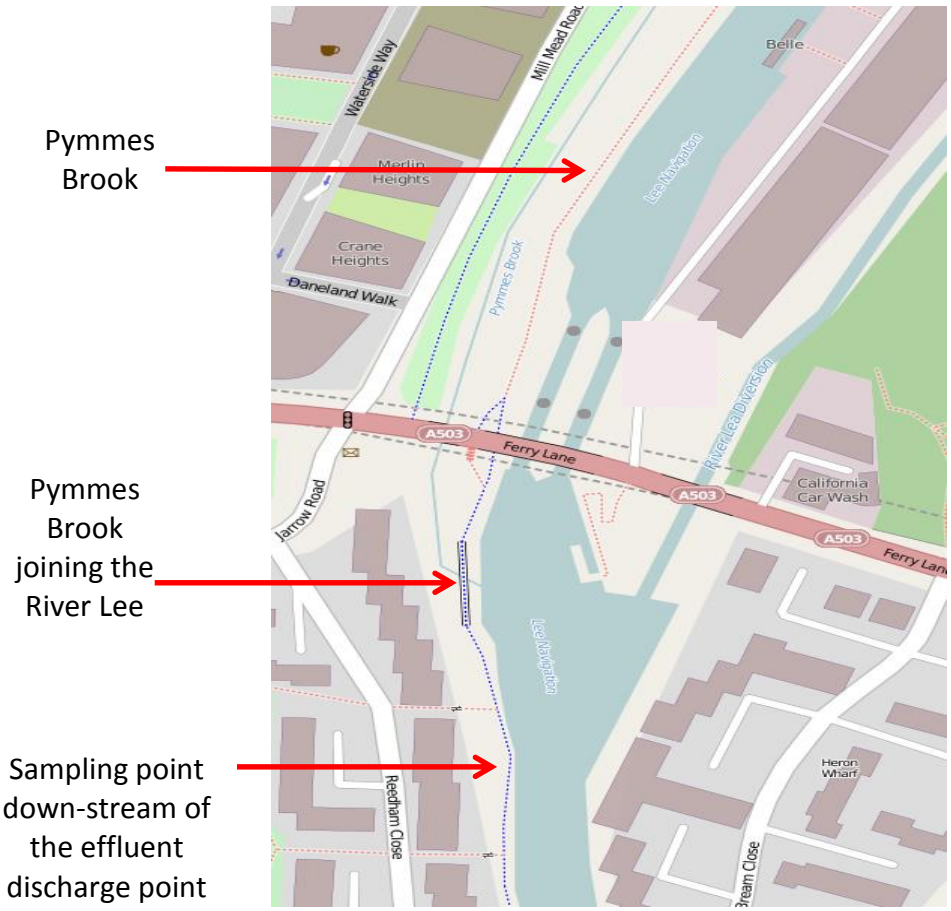


Map source: OpenStreetMap - Creative Commons-Share Alike License [CC-BY-SA]

Figure 4-3: Map of the lower Lee catchment showing the locations of the surface water sampling points relative to the WWTP.

For each surface water sampling point a total of 20 L was collected and fully mixed on return to the laboratory. At each of the wastewater treatment process sampling points, a total of 7.5 L was collected. Samples were filtered on the day of collection. Acidified samples (to pH 2.5 using sulphuric acid) were stored at 4°C until extraction (within 4 days). Settled sewage, final treated effluent and surface water were collected on six occasions between February

2011 and February 2012. Additionally screened sewage was collected on three occasions between February 2011 and February 2012.



Map source: OpenStreetMap - Creative Commons-Share Alike License [CC-BY-SA]

Figure 4-4: Map showing in more detail the location of the sampling point located down-stream of the WWTP effluent discharge.

4.2.4 Water quality parameter analysis

pH and total suspended solids (TSS) were measured for each sample. TSS was determined according to the procedure given in the standard methods for the examination of water and

wastewater (American Public Health Association (APHA), 1992). Filter papers (Whatman 90 mm GF/C) were heated in an oven (105 °C) until dry then placed in a desiccator until cool. The filter papers were then weighed. Fully mixed samples were passed through the filter papers under vacuum. The filter papers were placed in the oven overnight at 105 °C and re-weighed to constant weight after cooling in a desiccator. Each sample was analysed in triplicate. The suspended solids concentration was calculated using Equation 4-1.

Equation 4-1:

$$TSS\ (mg/L) = \frac{F^2 - F^1}{V}\ x\ 1000$$

Where:

F^2 = the weight of dried filter paper after filtration (mg)

F^1 = the weight of dried filter paper before filtration (mg)

V = volume of the sample filtered (mL)

4.2.5 Analytical method to determine target pharmaceutical concentrations

4.2.5.1 Extraction of water samples

Solid phase extraction (SPE) was performed using Strata™ X cartridges which were first conditioned with 6 mL methanol (LC-MS grade), equilibrated with 6 mL LC-MS water and then 6 mL LC-MS water with the pH adjusted to 2.5 (sulphuric acid). Samples were percolated (100 mL, 200 mL, 200 mL and 1000 mL for screened sewage, settled sewage,

final treated effluent and surface waters respectively) through the conditioned cartridges at an approximate flow rate of 2 mL/min using a vacuum extraction manifold (Phenomenex, UK). Larger volumes of surface water were extracted to provide the concentration required to be able to detect the lower levels of pharmaceuticals expected in these samples. To release the pharmaceuticals, the sorbent was washed with 6 mL water and then with 6 mL 5 % (v/v) methanol in water containing 2 % acetic acid and dried under vacuum for at least 10 minutes prior to elution with 2 % ammonium hydroxide in methanol (3 x 3 mL). The resulting combined extract was evaporated under a gentle nitrogen stream using a TurboVap® (Biotage, Sweden) at 30°C and reconstituted with 1.0 mL 5% (v/v) methanol/ultrapure water, before transferring to 0.2 µm nylon Mini-UniPrep® filter vials (Whatman Ltd, UK) for subsequent analysis.

4.2.5.2 Instrumental analysis

Analysis of the extracts was performed using reverse phase high performance liquid chromatography mass spectrometry (LC-MS) with electrospray ionization in positive (+ve) selective ionization mode (SIM) using a Shimadzu LC2010 instrument. The selected pharmaceuticals were separated using an Ascentis® Express 2.1 mm x 50 mm C18 column (Sigma Aldrich, UK). Amoxicillin, bezafibrate, carbamazepine, ciprofloxacin and clarithromycin were separated using mobile phases of LC-MS water acidified with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1 % formic acid (mobile phase B). The solvent gradient started at 5% B and reached 67% B in 20 minutes before increasing

to 95% B for 5 minutes and then returning to 5% B for 10 minutes. The column was maintained at 30 °C with a flow rate of 0.2 mL/min and an injection volume of 10 µL.

The developed analytical method was not applied to vancomycin and cefpodoxime. Initially, these antibiotics were not selected for this study because they are prescribed in very low quantities and therefore not expected to be present in environmental waters at detectable levels. Furthermore, reports on the presence of these compounds in environmental studies are rare (see Section 4.1.1). However whilst conducting the work for Chapter 5, it was decided it was important to look at the susceptibility of faecal bacteria to vancomycin and cefpodoxime as these are critically important for human medicine (see Chapter 5, Section 5.1.2).

4.2.6 Validation of the analytical method to determine target pharmaceuticals in environmental waters

The target compounds were monitored using their parent ion ($[M-H]^+$) in positive selective ion monitoring (SIM) mode. Quantification of the compounds of interest was performed using the standard addition method in which different concentrations were spiked into separate aliquots of each sample. The analyte concentration was then determined by linear regression. At least four concentration points were used to check the linear range of the method. To assess the overall impact of sample preparation and matrix effects on the measurement of the target compounds, spiking experiments were performed and the recovery of the spiked amount calculated. Screened sewage, settled sewage, final treated effluent and surface water were spiked with 3000, 1500, 1000 and 240 ng/L of each target

compound respectively. The concentration of targets used for the spiking experiments were selected based on those reported in literature (see Table 4-2 and Table 4-3). To evaluate the method repeatability (precision) for the individual compounds, the separate extractions and analyses of wastewater and surface water were carried out in triplicate.

The instrumental detection limit (IDL) was determined using external standards (HPLC grade pharmaceutical compounds dissolved in 5 % v/v methanol/LC-MS water). The IDL is defined as the analyte concentration that gives a signal to noise ratio of > 3 and refers to the limit of detection determined using clean solutions (external standards). The method limit of detection (MLOD) was estimated using real samples (processed through the entire analytical method) and is defined as the minimum concentration of analyte that gives a signal to noise ratio of > 3 in a given matrix. It was difficult to determine the method limit of detection (MLOD) for effluent and surface waters, as the samples already contained the compounds of interest.

4.2.7 Prediction of pharmaceutical consumption

The Health and Social Care Information Centre - Prescribing and Primary Care Services (2012) maintains a database based on NHS prescription services in England. The database, which is produced annually, only includes the quantities of pharmaceuticals dispensed in the community (community pharmacists, appliance contractors, dispensing doctors, and items personally administered by doctors). The consumption of the selected pharmaceuticals for this study in England and in the catchment area of the WWTP under investigation was calculated based on the information available in this database.

4.3 Wastewater and surface water analysis

4.3.1 Water quality parameters

The pH and total suspended solids (TSS) determined for screened sewage, settled sewage, final treated effluent and the receiving surface waters are presented in Table 4-4. Measurements were made in triplicate on each of the six sampling occasions.

Table 4-4: Water quality parameters (mean ± standard deviation) measured for each sampling point for each sampling occasion.

Sample	pH	TSS (mg/L)
Screened sewage	6.8 ± 0.2	433.6 ±147.9
Settled sewage	6.9 ± 0.4	316.2 ± 102.4
Final treated effluent	7.1 ± 0.5	50.2 ± 21.9
Down-stream	7.8 ± 0.4	124.8 ± 52.5
Up-stream	7.6 ± 0.5	81.1 ± 33.9

Up- and down-stream refer to surface water collected up- and down-stream relative to the WWTP final effluent discharge point. Values are a mean of triplicate measurements made on 6 sampling occasions (18 measurements in total).

On average, a high proportion of suspended solids were removed during the full wastewater treatment process (> 85 %). Higher TSS values were observed in the surface water down-stream of the wastewater treatment plant (WWTP) treated effluent discharge point compared to up-stream. In addition, TSS values were higher in surface water than in the final treated effluent. The surface water pH values measured are within the range (≥ 6.0 – ≤ 9.0) expected in rivers in the UK for the support of biota (UK Technical Advisory Group on

the Water Framework Directive (UKTAG), 2008). Biological treatment of wastewater occurs generally at neutral pH (Bitton, 1944) and in this study the wastewater pH values were within the range 6.8 to 7.1.

4.3.2 Detection of pharmaceuticals in environmental waters with LC-MS^n

The instrumental detection limits (IDL) determined using the method given in Chapter 4, Section 4.2.6 for each of the target pharmaceuticals are given in Table 4-5.

Table 4-5: Physicochemical properties of target pharmaceuticals and instrumental detection limits (IDL).

Pharmaceutical	pKa	Log K_{ow}	Solubility (mg/L)	IDL (µg/L)
Amoxicillin	2.4, 7.4, 9.6[a]	0.87[c]	3740[c]	12
Bezafibrate	3.6[a]	4.25[a]	1.5[c]	0.3
Carbamazepine	13.9[a]	2.45[a]	18.0[b]	0.2
Ciprofloxacin	6.09, 8.74[b]	0.28[b]	30,000[b]	0.8
Clarithromycin	8.99[a]	3.16[a]	0.3[c]	0.2
Vancomycin	2.6, 7.2, 8.6[c]	1.1[c]	225.0[c]	ND
Cefpodoxime	3.22[c]	0.05[c]	185.0[c]	ND

pKa = acid dissociation constant, Log K_{ow} = Octanol water coefficient, [a] Ternes et al. (2008), [b] Wexler Philip (2001), [c] Wishart et al. (2008). ND – not determined in this study

The IDLs were not sensitive enough to enable the direct detection of the target compounds in environmental waters as they are typically present at ng/L concentrations (see Table 4-2 and Table 4-3. In addition, environmental waters are complex matrices that contain components that may interfere with the analytical measurement by matrix effects. Therefore, the concentration/purification step provided by solid phase extraction was

included to improve method sensitivity. The definition of instrumental detection limit (IDL) and method limit of detection are given in Chapter 4, section 4.2.6, page 78.

In LC-MS^n analysis, the chromatographic retention time may shift significantly due to matrix effects. Additional matrix components may mask the signal from the analytes of interest by raising the chromatographic baseline and with electrospray ionisation, matrix components may suppress or enhance ionisation by competing for charged sites on electrospray droplets (Gracia-Lor et al., 2010). Matrix effects can also cause false positive identification due to other sample components having similar mass to charge ratios (m/z). The most direct means of obtaining the appropriate method sensitivity and method selectivity for the detection of pharmaceuticals in environmental waters is through the reduction of matrix components prior to LC-MS^n analysis by applying a selective extraction and improved sample clean-up (Fatta et al., 2007). In this work, solid phase extraction (SPE) was selected to concentrate the analytes of interest and reduce matrix interferences in order to improve method sensitivity and selectivity. However, the different physicochemical properties of amoxicillin, bezafibrate, carbamazepine, ciprofloxacin and clarithromycin (Table 4-5) presented a challenge when selecting the SPE parameters required to achieve an efficient extraction of all selected compounds from the different environmental water matrices.

Amoxicillin is relatively unstable in aqueous solutions and its degradation is catalysed by both acids and bases which are commonly used to optimise solid phase extraction recoveries. Castiglioni et al. (2005) and Watkinson et al. (2009) have reported low amoxicillin recoveries (36 and 29 % respectively), making it difficult to accurately and

reproducibly detect this compound in wastewaters. In this study, amoxicillin could not be detected following the solid phase extraction of collected water samples. Bezafibrate is an acidic drug that is the most hydrophobic of the selected pharmaceuticals as demonstrated by the chromatographic retention time (given in Table 4-8) and octanol water coefficient (Table 4-5). Ciprofloxacin is zwitterionic and hydrophilic, clarithromycin is a basic hydrophobic compound and carbamazepine (a neutral drug) is moderately hydrophobic compared to bezafibrate. A polymeric sorbent (Strata™ X) was therefore chosen for the extraction process as it retains acidic, basic and neutral compounds. The neutral polar functionalised styrene reversed phase polymer exhibits hydrophobic, hydrogen-bonding, and aromatic retention mechanisms and has been successfully employed in previous studies focused on the extraction of pharmaceuticals from environmental waters (Babić et al., 2010; Lacey et al., 2008).

The sample pH and elution solvent characteristics were the two SPE parameters that were manipulated to ensure the maximum recovery of target compounds using the polymeric sorbent, whilst minimising other matrix components that may interact with the sorbent reducing the interaction sites for the target analytes and subsequently reducing detection. Generally in other studies, the pH of the samples have been adjusted to favour either the dissociated or the non-dissociated forms to ensure optimal retention of the compounds of interest to the sorbent without retention of other matrix components. The compounds of interest are then eluted with a solvent that is efficient at reducing the interaction between the sorbent and the target analytes.

For macrolides and fluoroquinolones, Senta et al., (2008) achieved recoveries (> 60 %) with a low sample pH using either Strata™ X or Oasis® HLB polymeric sorbents and a basic methanol elution solvent. Renew et al., (2004) achieved relative extraction recoveries (the peak area of target compound compared to the peak area of an internal standard) of 90-129% for fluoroquinolones from final wastewater treatment effluents utilising two different sorbents in tandem (anionic followed by polymeric), a sample pH of 2.5 and elution with a buffered (H_3PO_4) methanol solution.

Babić et al. (2010) used Strata™ X cartridges for the extraction of fluoroquinolones and macrolides achieving extraction recoveries of > 50 % when samples were adjusted to pH 4 and target analytes were eluted with methanol. For the extraction of macrolides from wastewater effluents, Oasis® HLB polymeric sorbents were used by Pedrouzo et al. (2008). Samples were adjusted to pH 7 with sodium hydroxide and eluted with a basic methanol solution. However, extraction recoveries for macrolides were < 50%. McArdell et al., (2003) achieved relative extraction recoveries (compared to an internal standard) of 81% ± 9 for clarithromycin from wastewater effluents using Lichrolute reversed phase and copolymer sorbents, a sample pH adjusted to 7 and elution with methanol. Oasis® HLB polymeric sorbent and a sample pH adjusted to 10 have been utilised by Vieno et al. (2006) to extract a range of pharmaceuticals with different physicochemical properties including carbamazepine and ciprofloxacin. This approach resulted in extraction recoveries of 64% for ciprofloxacin and 94% for carbamazepine from wastewater influent. Bezafibrate was extracted using a mixed mode cationic polymeric sorbent (Oasis® MCX) and a sample pH of 2 by Lindqvist et al., (2005). The water extracts were eluted with acetone and absolute

recoveries (area of analyte of interest compared to external standards) of 64% were achieved.

In this work, the sample pH was adjusted to pH 2.5 in order to suppress the dissociation of the carboxylic functional groups present in bezafibrate and ciprofloxacin and therefore to favour the hydrophobic interactions of these analytes with the polymeric sorbent (Senta et al., 2008). For acidic moieties, reducing the sample pH below the pKa will favour the non-dissociated form making them less soluble. According to other studies, carbamazepine is not affected by adjusting sample pH as it is a neutral compound (Vieno et al., 2006) and a better extraction of clarithromycin is achieved at a lower sample pH (Senta et al., 2008; Göbel et al., 2007). Increasing the retention of the analytes to the sorbent allowed for a more aggressive clean-up of the matrix components with an acidified methanol and water solution prior to elution. A 2% ammonium hydroxide in methanol solution was used to disrupt the hydrophobic interactions for elution and desorb the target analytes. The percentage recovery of the target compounds from each water matrix was assessed using spiking studies. Triplicate aliquots of each sample matrix were spiked with a known amount of each target analyte and the % recovery calculated according to Equation 4-2. The percentage recoveries identify the losses resulting from SPE extraction.

The spiked amounts were chosen to be relevant to each sample matrix and to ensure the concentrations were within the method linear range. The recoveries of target analytes calculated for screened sewage (spiked at 3000 ng/L), settled sewage (spiked at 1500 ng/L),

final treated effluent (spiked at 1000 ng/L) and surface waters up- and down-stream of the treated effluent discharge point (spiked at 240 ng/L) are shown in Table 4-6.

Recoveries exceeded 60% for the target compounds in the five matrices and the precision (% RSD) of triplicate extractions were typically < 15%. Higher recoveries were obtained for carbamazepine (75–98%) and bezafibrate (75-98%) compared to ciprofloxacin (61–87%) and clarithromycin (60-96%). Generally, higher recoveries resulted from treated effluent (87-98%) and up-stream surface waters (75–93%) for all compounds compared to screened sewage (60-87%), settled sewage (60-80%) and surface water down-stream of the effluent discharges (60-95%). These results are consistent with the treated effluents and up-stream waters generally containing less organic matter. Spiking experiments showed a more efficient extraction of bezafibrate was achieved from down-stream surface water (95 %) compared to up-stream (75 %). This is surprising as the surface water down-stream from the effluent discharge point contains elevated suspended solids (Table 4-4) which will hinder the extraction efficiency. However, this could be due to the elevated concentrations of bezafibrate already present in the down-stream surface water matrix compared to up-stream which were not accounted for in Equation 4-2. Amoxicillin could not be reliably detected following sample extraction and is therefore not included in Table 4-6.

Equation 4-2:

$$\%\, recovery\ = \left(\frac{Pre\ Spike}{Post\ Spike}\right) 100$$

Where:

$Pre\ spike$ = The amount of the target compound measured in an aliquot of sample spiked with a known amount of the target prior to sample preparation

$Post\ spike$ = The amount of the target compound measured in an aliquot of the same sample spiked with a known amount of the target just before LC-MS^n injection

Table 4-6: SPE recoveries and extraction precision (% RSD) determined for target pharmaceuticals in the different environmental water matrices investigated in this study.

	% Recovery (% RSD)				
	Screened sewage	Settled sewage	Final treated effluent	Up-stream	Down-stream
Bezafibrate	87 (10)	80 (10)	91 (9)	75 (6)	95 (13)
Carbamazepine	84 (10)	75 (1)	98 (6)	93 (6)	73 (3)
Ciprofloxacin	61 (4)	66 (15)	87 (10)	83 (11)	67 (6)
Clarithromycin	60 (8)	60 (4)	96 (6)	82 (11)	60 (3)

% Recovery and relative standard deviation (% RSD) are mean values calculated from 3 replicate extractions.

Variations in the recoveries of the target compounds can affect the accuracy of quantification and to reduce this there are a number of compensatory approaches that can be used. These include calibration with matrix matched external standards, dilution of the extract from the complex matrix, the application of internal standards, improvement of chromatographic separation and the use of the standard addition technique. It is difficult to match the matrix of an environmental water sample due to the complex array of unknown constituents and therefore this approach was not used in this work. Dilution of the samples

was not used either as it would decrease the sensitivity of the method as shown in a study of fluoroquinolones in settled sewage and final treated effluent (Lee et al., 2007).

The use of internal standards is a common method employed to compensate for extraction recoveries and for the quantification of pharmaceuticals in environmental waters (Göbel et al., 2007; McArdell et al., 2003). An internal standard should not be naturally present in the environmental waters and should have similar physicochemical properties to the compounds of interest and therefore similar chromatographic retention times, to adequately compensate for matrix effects. Generally, isotope labelled compounds are used (surrogate standard) and spiked into the sample before SPE to compensate for sample preparation losses as well as matrix effects. For the analysis of pharmaceuticals with different physicochemical properties, a representative internal standard would be required for each class or group of pharmaceutical for accurate quantification (Hernández et al., 2007). The cost implications ruled this method out as a quantification technique in this study.

Standard addition was utilised to quantify target analytes and compensate for sample extraction losses. A disadvantage of this method is that it is an extrapolation method and is less precise than interpolation methods. Therefore the analyte concentration (the extrapolated value) can have an elevated standard deviation. The standard deviation of the extrapolated value (s_{XE}) can be calculated using Equation 4-3 employing the standard deviation of the y residuals ($s_{y/x}$) which is calculated using Equation 4-4.

Equation 4-3:

$$s_{XE} = \frac{s_{y/x}}{b}\left\{\frac{1}{n} + \frac{\bar{\gamma}^2}{b^2 \sum_i (x_i - \bar{x})^2}\right\}^{\frac{1}{2}}$$

Where:

b = gradient of regression line

n = sample size

$\bar{\gamma}$ = arithmetic mean of y residuals

$\bar{x}$ = arithmetic mean of x values

x_i = Individual x value

Equation 4-4:

$$s_{y/x} = \left\{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-2}\right\}^{\frac{1}{2}}$$

Where:

$\hat{y}_i$ = values calculated from the regression line corresponding to the individual x values

y = Individual y measurement

Examples of the standard deviations of the extrapolated derived analyte concentrations are given in Table 4-7.

Table 4-7: Examples of the target pharmaceutical concentrations in environmental waters quantified by standard addition and the standard deviation of the extrapolated value (±)

	Screened sewage	Settled sewage	Final treated effluent	Down -stream	Up -stream
Carbamazepine (ng/L)	1214.2 ± 152.2	1501.9 ± 407.9	1010.0 ± 254.6	251.4 ± 46.2	128.8 ± 30.5
Bezafibrate (ng/L)	1693.8 ± 362.2	1341.8 ± 259.6	246.9 ± 25.32	103.6 ± 34.6	30.79 ± 21.5
Ciprofloxacin (ng/L)	2539.9 ± 161.1	3479.8 ± 594.2	490.0 ± 143.1	149.8 ± 38.4	<MLOD
Clarithromycin (ng/L)	<MLOD	783.5 ± 478.1	460.9 ± 232.2	28.6 ± 8.2	< MLOD

< MLOD not detected in these samples at concentrations above the method limit of detection. Down-stream and up-stream = relative to the treated effluent discharge point.

Peak resolution is important to aid the identification of the analytes and to prevent false positive identification of other analytes with similar m/z values. In this study the adequate separation of the target analytes was achieved using a combination of aqueous and acetonitrile mobile phases acidified with formic acid. An example of the chromatographic separation of the target analytes is shown in Figure 4-5. Formic acid was added to the mobile phases to suppress interaction of the basic amino moiety of ciprofloxacin with the column silanol groups that can cause peak tailing, effecting peak asymmetry and reducing sensitivity. Acidic additives are known to promote protonation of basic functionalities and as a result can enhance the signal in the electrospray ionisation source by operating in positive mode (Lee et al., 2007).

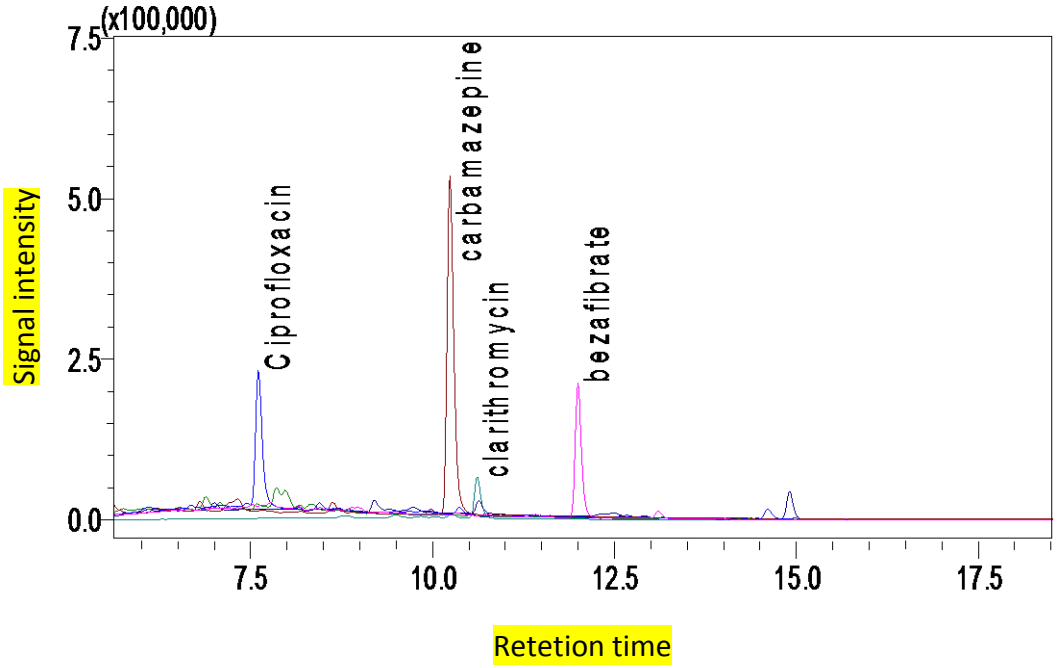


Figure 4-5: Mass spectrometer chromatogram showing positive selective ion monitoring of target pharmaceuticals in settled sewage.

In addition, formic acid (0.1 %) was also used as it is volatile and easily removed during electrospray ionisation. Trifluoroacetic acid was trialled as a mobile phase additive but this suppressed ionisation of the analytes and using ammonium acetate resulted in poor peak asymmetry for ciprofloxacin and therefore its use was discontinued. Some pharmaceuticals with basic and acidic functional groups can either be protonated or depronated for detection in positive or negative electrospray ionisation modes. Bezafibrate is an example that is sensitive in both modes (Gros et al., 2006). However, in this study positive selective ionisation mode (SIM) was the most sensitive for all compounds. The positive ions monitored in addition to retention times, for identification and quantification, are shown in Table 4-8.

Table 4-8: Retention times and parent ion $[M-H]^+$ for the target pharmaceuticals.

Compound	LC-MS retention time (minutes)	$[M+H]^+$
Bezafibrate	11.9	362
Carbamazepine	10.1	237
Ciprofloxacin	7.6	332
Clarithromycin	10.6	748

The performance of the method developed for the determination of bezafibrate, carbamazepine, ciprofloxacin and clarithromycin in different environmental water matrices is summarised in Table 4-9. At least four point calibrations were used for the standard addition quantification of target analytes (in the ranges 500 – 6000 ng/L, 250 - 4000 ng/L, 250 – 4000 ng/L and 40 - 500 ng/L for screened sewage, settled sewage, final treated effluent and surface waters respectively). Overall, good linearity was obtained for target compounds in the different matrices, with correlation coefficients (r^2) generally > 0.94 for the concentration ranges expected in each matrix. Although the method developed in this work was not as sensitive as methods reported in other studies for individual compounds, it was suitable to detect the selected compounds in all the matrices. The method limit of detection (MLODs) ranged between 5 ng/L for bezafibrate in surface waters to 500 ng/L for ciprofloxacin in screened sewage (see Table 4-9).

Table 4-9: Linearity of calibration method and method limit of detection (MLOD) determined for the selected pharmaceuticals in different environmental water matrices.

Pharmaceutical	Linearity (r^2) MLOD (ng/L)				
	Screened sewage	Settled sewage	Final treated effluent	Up-stream	Down-stream
Bezafibrate	0.9901	0.9859	0.9941	0.9677	0.9805
	~ 80	~ 40	~ 20	~ 5	~ 10
Carbamazepine	0.9929	0.9686	0.9790	0.9915	0.9406
	~ 60	~ 60	~ 20	~ 5	~ 10
Ciprofloxacin	0.9934[a]	0.9760[a]	0.9590[b]	0.9901[b]	0.9700[a]
	~ 450	~ 150	~ 30	~ 25	~ 40
Clarithromycin	0.9867	0.9450[b]	0.9943	0.9730	0.9851
	~ 500	~ 150	~ 40	~ 10	~ 10

MLOD was estimated for each sample matrix at a signal to noise ratio 3. 6 point calibration except where indicated. [a]4 point calibration. [b]5 point calibration.

4.3.3 Occurrence of pharmaceuticals in wastewaters and surface waters

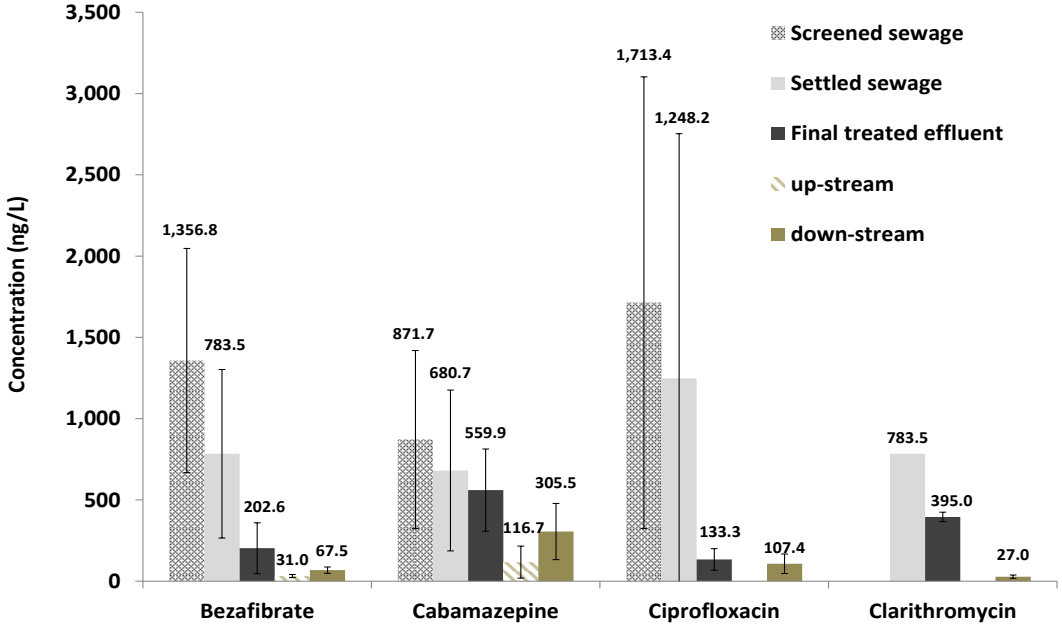
The developed analytical method for the detection of bezafibrate, carbamazepine, ciprofloxacin and clarithromycin was applied to the analysis of wastewaters sampled at three different treatment points in a large urban wastewater treatment plant and in the surface waters both up- and down-stream realtive of the treated effluent discharge point. The ranges of the target pharmaceutical concentrations observed at each sampling point are presented in Table 4-10.

Table 4-10: Pharmaceuticals detected in wastewater sampled at different points throughout the treatment process and receiving waters between February 2011 and February 2012.

Compound		Screened sewage (n = 3)	Settled sewage (n = 6)	Treated effluent (n = 6)	Up-stream (n = 5)	Down-stream (n = 5)
Bezafibrate	Range (ng/L)	628-2147	121-1342	39-411	22-40	44-88
	Freq (%)	100 %	83 %	100 %	60 %	100 %
Carbamazepine	Range (ng/L)	369-1460	206-1502	47-1010	39-251	156-585
	Freq (%)	100 %	100 %	100 %	80 %	100 %
Ciprofloxacin	Range (ng/L)	731-2696	297-3478	44-229	Nd	65–150
	Freq (%)	67 %	67 %	83 %	0 %	40 %
Clarithromycin	Range (ng/L)	Nd	[a]783	57-598	Nd	19-34
	Freq (%)	0 %	17 %	83 %	0 %	60 %

Freq (%) = frequency of detection. n = number of sampling occasions. Nd = not detected on any sampling occasion. Up- and Down- = in surface water from the discharge point. [a]Only detected on one occasion and therefore no range given.

Not all target compounds were detected in all samples as indicated by the frequencies of detection identified in Table 4-10. Figure 4-6 displays the mean concentrations (± standard deviation) of each compound at each sampling point. Where the relevant monitoring data is available, the general trend observed is a decrease in target pharmaceutical concentrations throughout the wastewater treatment process (Figure 4-6). The levels of the target pharmaceuticals were highly variable at each sampling point, particularly for ciprofloxacin in the screened and settled sewage. However, the levels (mean ± standard deviation) of bezafibrate (1356.8 ± 689.6 ng/L) and ciprofloxacin (1713.4 ± 1389.4 ng/L) observed in the screened sewage were higher compared to carbamazepine (871.7 ± 547.4 ng/L).



Bars indicates the mean concentration (ng/L) detected from all sampling occasions
No bar indicates the pharmaceutical was not detected in this matrix
Data labels above the bars indicate the mean value
Error bars indicate standard deviation
No error bars indicate only one measurement

Figure 4-6: Mean concentrations of target pharmaceuticals detected in different samples

In the settled sewage, the concentrations of target pharmaceuticals were within the range of 680.7 ± 494.4 (carbamazepine) to 1248.2 ± 1505.1 (ciprofloxacin) ng/L, indicating that small reductions occurred during the primary sedimentation process. Clarithromycin could only be detected in one settled sewage sample (783.5 ng/L) and was not detected in the screened sewage.

Noticeably reduced bezafibrate (202.6 ± 156.5 ng/L) and ciprofloxacin (133.3 ± 252.8ng/L) levels were observed in the final treated effluent samples. Conversely, carbamazepine was detected at only modestly reduced concentrations in the final treated effluents (559.9 ±

66.4 ng/L) compared to the screened and settled sewage. The frequency of clarithromycin detection was greater for the final treated effluent samples (83 %) compared to other sampling points ($\leq$ 17 %) in the wastewater treatment process.

Bezafibrate, carbamazepine, ciprofloxacin and clarithromycin were all detected in the surface water down-stream of the WWTP effluent discharge point but whereas bezafibrate and carbamazepine were consistently detected ciprofloxacin and clarithromycin were found intermittently. Bezafibrate and carbamazepine were also detected in the surface water up-stream of the wastewater treatment plant effluent discharge point. However, ciprofloxacin and clarithromycin were not detected at this location (Table 4-10).

4.3.4 Reduction of pharmaceuticals through wastewater treatment processes.

The reduction of target compounds following primary sedimentation, following activated sludge treatment and the overall reduction efficiency of the wastewater treatment process were calculated for each sampling occasion using Equation 4-5, Equation 4-6 and Equation 4-7, respectively. The calculated percentage reductions are presented in Table 4-11. The use of pharmaceutical concentrations in these calculations assumes that the flows remain unchanged as the compounds pass through the sewage treatment plant.

Equation 4-5:

$$\text{Reduction following primary sedimentation (\%)} = \frac{\text{screened sewage-settled sewage}}{\text{screened sewage}} \times 100$$

Equation 4-6:

$$\text{Reduction following activated sludge treatment (\%)} = \frac{\text{Settled sewage-final treated effluent}}{\text{settled sewage}} \times 100$$

Equation 4-7:

$$\text{Overall WWTP reduction (\%)} = \frac{\text{screened sewage-final treated effluent}}{\text{screened sewage}} \times 100$$

Where:

Screened sewage = measured concentration in screened sewage (ng/L)

Settled sewage = measured concentration in settled sewage (ng/L)

Final treated effluent = measured concentration in final treated effluent (ng/L)

Low and variable mean percentage reductions (< 22 %) were observed for bezafibrate, carbamazepine and ciprofloxacin during primary sedimentation. In addition, Figure 4-7 shows some negative reductions were obtained on some sampling occasions due to the detection of higher concentrations in the settled sewage compared to the screened sewage. Clarithromycin could not be detected in the screened sewage and hence the reduction of clarithromycin following primary sedimentation could not be calculated. Following activated

sludge treatment, the individual percentage reductions were high for bezafibrate (within the range: 40.7 – 90.8 %) and ciprofloxacin (within the range: 40.9 – 95.8 %). Conversely, lower and highly variable reductions were obtained for carbamazepine (within the range: -96.3 – 46.3 %) with negative reductions observed on two sampling occasions (Figure 4-7).

Table 4-11: Mean (n = 6) percentage reductions (mean ± standard deviation) of pharmaceuticals at different treatment stages of the WWTP.

Pharmaceutical	Primary sedimentation % reduction (n = 3)	Activated sludge % reduction (n = 6)	Overall % reduction (n = 3)
Bezafibrate	21.9 ± 43.3	73.5 ± 20.6	89.7 ± 5.7
Carbamazepine	20.0 ± 29.7	-4.7 ± 52.9	22.5 ± 11.9
Ciprofloxacin	15.1 ± 62.4	77.1 ± 24.6	94.3 ± 0.5
Clarithromycin	nc	[a]59.5	nc

nc = not calculated. [a] Only detected on one occasion

A reduction of 59.5 % was obtained for clarithromycin following activated sludge treatment but this was based on the detection of clarithromycin in one settled sewage sample. Overall, the target pharmaceuticals were not completely removed from the wastewater treatment process (Table 4-11). High overall reductions (mean ± standard deviation) were observed for bezafibrate (89.7 ± 5.7 %) and ciprofloxacin (94.3 ± 0.5 %). Whereas, the overall reduction for carbamazepine was much lower (22.5 ± 11.9 %).

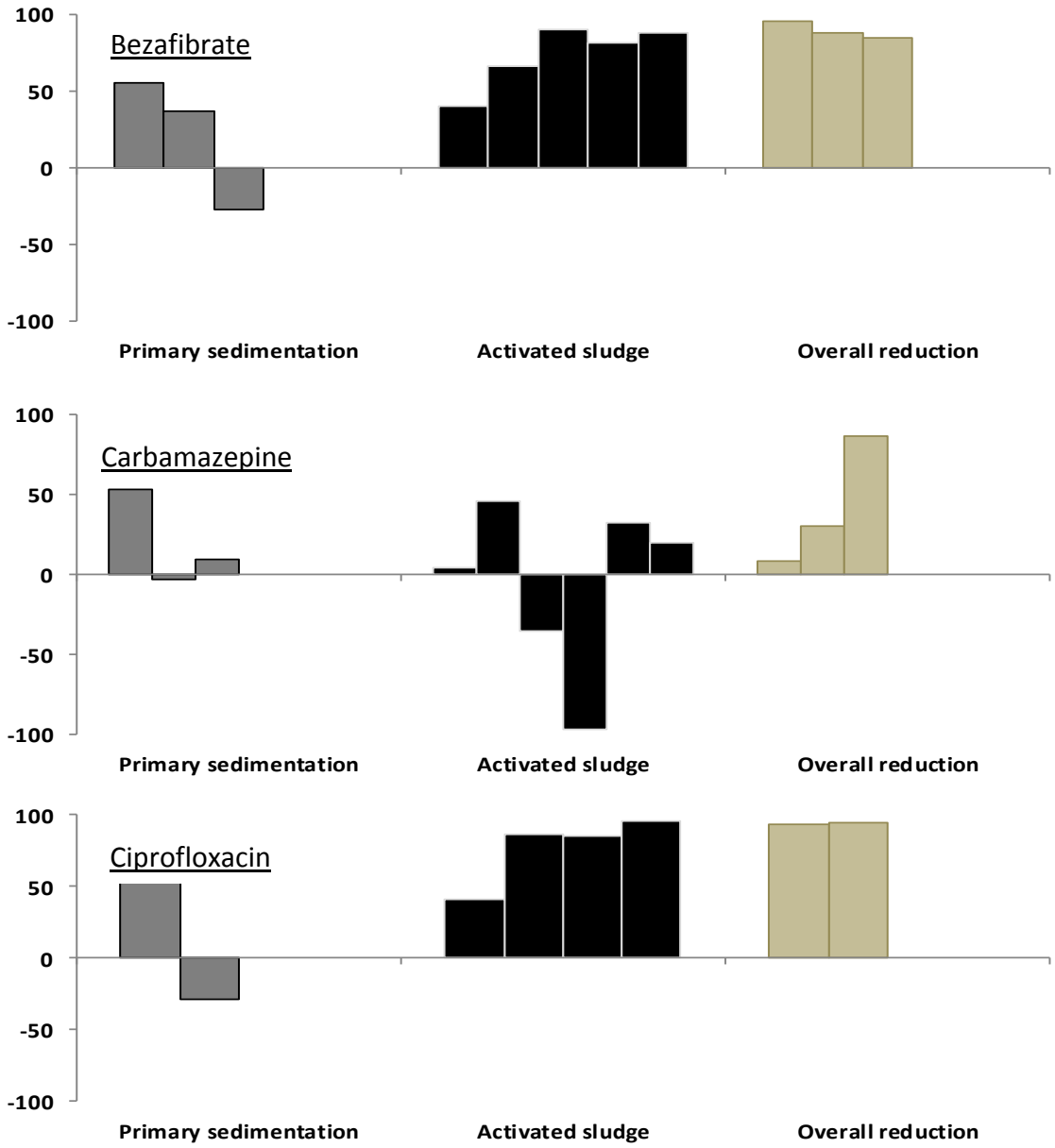


Figure 4-7: The percentage reduction of bezafibrate, carbamazepine and ciprofloxacin calculated for each sampling occasion; following primary sedimentation (n = 3), activated sludge treatment (n = 6) and the overall reduction (n = 3). Percentage reductions have not been reported where compounds could not be detected in wastewater samples at levels above the MLOD (signal to noise > 3).

4.3.5 Comparison of predicted and measured influent concentrations

Prescription cost analysis (PCA) data collated by The Health and Social Care Information Centre - Prescribing and Primary Care Services (2012) was used to estimate the quantities of selected pharmaceuticals (bezafibrate, carbamazepine, ciprofloxacin and clarithromycin) prescribed in England per annum and are given in Table 4-12.

Table 4-12: Prescription quantities (England, 2011) for selected pharmaceuticals and predicted wastewater influent concentrations

Pharmaceutical	Tonnes a^{-1} (2011)	$PEC_{influent}$ (ng/L)
Bezafibrate	7.0	~ 1,400
Carbamazepine	43.0	~ 8,000
Ciprofloxacin	7.2	~ 1,400
Clarithromycin	12.0	~ 2,000

Tonnes a^{-1} = tonnes prescribed per annum, $PEC_{influent}$ = Predicted environment concentration for the WWTP catchment influent ignoring any transformation or metabolic processes.

The quantities prescribed in 2011 varied between 7.0 (bezafibrate) and 43.0 (carbamazepine) tonnes and highlight that there are considerable differences in the types and quantities prescribed each year which will influence the levels of these compounds finding their way to urban wastewaters. The pharmaceuticals selected for this study are only available on prescription in England and therefore the prescription data provided by the NHS is a good indicator of the quantities used. The low prescribed levels of vancomycin and cefpodoxime meant that these compounds were not included when selecting pharmaceuticals for monitoring in the environmental waters in this work. These low

prescription levels are consistent with these compounds being rarely reported in environmental waters compared to compounds such as bezafibrate and carbamazepine.

By scaling down the estimated pharmaceutical prescription quantities for England to those expected for the population within the catchment of the studied WWTP (population 870,000), predicted influent concentrations ($PEC_{influent}$) entering the WWTP have been estimated using Equation 4-8 and are given in Table 4-12.

Equation 4-8:

$$\frac{\left(\frac{Pop_{catchment}}{Pop_{England}}\right) \times Q}{(DWF \times 365)} = PEC_{influent}$$

Where:

$Pop_{catchment}$ = Population of catchment (~ 870,000)

$Pop_{England}$ = Population of England (~ 52 million)

Q = Quantity estimated from PCA data (tonnes a^{-1})

DWF = Dry weather flow (typical 244,000 m^3 day^{-1})

$PEC_{influent}$ = Predicted environment concentration for the catchment wastewater influent without taking into account metabolism or transformation

The predicted influent concentrations ($PEC_{influent}$) for carbamazepine and clarithromycin are higher than the concentrations typically reported in the literature (see Table 4-2). Whilst the predicted bezafibrate and ciprofloxacin influent concentrations fall within the range of concentrations reported in literature (Table 4-2). However, the pharmaceuticals selected for

this study are typically administered orally or by injection and therefore will undergo metabolic processes within the body before excretion. Consequently, not all the pharmaceuticals will be excreted in the unchanged form.

Therefore, for a more realistic indication of the pharmaceutical load into urban wastewater treatment plants, the percentage of the pharmaceutical excreted as the unchanged compound needs to be considered. Taking into account the typical percentage of each of the selected pharmaceuticals excreted in the unchanged form, predicted environmental concentrations of the excreted unchanged pharmaceuticals have been calculated using Equation 4-9 for the influent to the WWTP under study ($PEC_{influent\text{-}unchanged}$).

Equation 4-9

$$PECinfluent_{\text{-unchanged}} = PECinfluent\ x\ \%\ excreted\ unchanged$$

The proportions of carbamazepine (< 10 %) and clarithromycin (20 %) excreted in the unchanged form are lower than for bezafibrate (50 %) and clarithromycin (50 %). When the proportions of bezafibrate, carbamazepine and ciprofloxacin excreted in the unchanged form are taken into account, the predicted influent concentrations ($PEC_{influent\text{-}unchanged}$) are consistent with their measured ranges in the screened sewage (Table 4-13). On the basis that only 20 % of clarithromycin is typically excreted in the original form, the estimated influent concentration for clarithromycin in the unchanged form (500 ng/L) explains why clarithromycin could not be detected above the analytical method quantification limit (500 ng/L) in the screened sewage.

132Table 4-13: Predicted influent concentrations (using typical excretion data) compared to those measured in screened sewage.

Pharmaceutical	Excreted unchanged (%) [a]	$PEC_{influent\text{-}unchanged}$ (ng/L)	$MEC_{screened\ sewage}$ (ng/L)
Bezafibrate	~ 50	~700	628-2147
Carbamazepine	~ 10	~800	369-1460
Ciprofloxacin	~ 50	~700	731-2696
Clarithromycin	~ 20	~500	(< 500)

MEC = Measured (range) environmental concentrations in screened sewage of WWTP studied for this work. $PEC_{influent\text{-}unchanged}$ = Predicted influent concentration of the unchanged pharmaceutical. Method limit of quantification given in parenthesis where targets were below detection limits. ND – Not detected using described method. [a] Wishart et al. (2008). Excreted unchanged values will vary depending on age and sex of patient.

4.3.6 Comparison of pharmaceutical levels in surface waters up- and down-stream of the WWTP treated effluent discharge point

The incomplete removal of pharmaceuticals in wastewater treatment processes poses problems for receiving waters as evidenced by the consistently increasing down-stream levels. The incomplete removal of bezafibrate, carbamazepine, ciprofloxacin and clarithromycin resulted in down-stream (mean ± standard deviation) concentrations of 67.5 ± 19.5, 305.5 ± 172.9, 107.4 ± 60.0 and 27.0 ± 10.0 ng/L respectively compared to 31.0 ± 9.4, 116.6 ± 98.5 ng/L for bezafibrate and carbamazepine up-stream (Figure 4-6). Ciprofloxacin and clarithromycin could not be detected in the surface water up-stream of

the effluent discharge point at concentrations greater than the method limit of detection given in Table 4-9 (25 and 10 ng/L for ciprofloxacin and clarithromycin respectively).

Figure 4-8 graphically presents the individual concentrations of bezafibrate, carbamazepine, ciprofloxacin and clarithromycin detected in all surface water samples collected during this study and also displays the action limit recommended by the European Medicines Agency (2006).

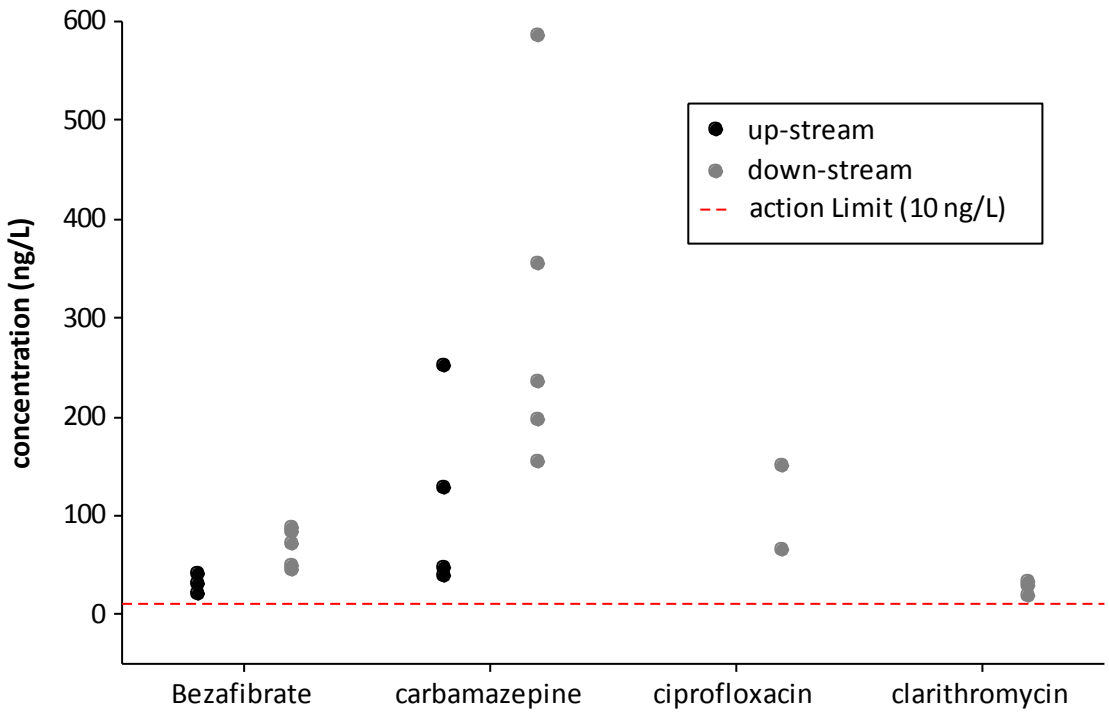


Figure 4-8: Interval plot presenting the individual concentrations of target pharmaceuticals in the surface waters both up- and down-stream of the treated effluent discharge point of the WWTP investigated in this study. The red dashed line indicates the action limit recommended by the European Medicines Agency (2006).

The presence of the target pharmaceuticals up-stream of the final treated effluent discharge point of the WWTP studied demonstrated the presence of additional sources of

pharmaceuticals higher up in the catchment. Interestingly, where the pharmaceuticals have been detected in the surface water samples, the concentrations exceed the action limit (10ng/L). Therefore if these pharmaceutical compounds were new to the market they would require experimental testing to evaluate their environmental fate and effects (see Section 2.5.4) prior to approval from drug regulatory agencies such as the European Medicines Agency (EMA, 2012).

4.4 Discussion

4.4.1.1 Detection of target pharmaceuticals in wastewater and surface waters

In this study a liquid chromatography mass spectrometry method has been employed to detect bezafibrate, carbamazepine, ciprofloxacin and clarithromycin in environmental waters. Initially an attempt to extract amoxicillin from environmental waters was made. However, amoxicillin is unstable in aqueous solutions and its hydrolysis is catalyzed in the acidic conditions which were used for sample extraction. Unfortunately, due to the different physico-chemical properties of pharmaceutical compounds, it is difficult to find method parameters that are suitable for the extraction and analysis of all selected analytes. However, analyte recoveries (Table 4-6), method limits of detection and linearity (Table 4-9) were considered adequate to detect bezafibrate, carbamazepine, ciprofloxacin and clarithromycin in wastewater and surface water samples.

4.4.1.2 Occurrence of target pharmaceuticals in wastewaters

The concentrations of the target pharmaceuticals detected in the screened sewage, settled sewage and final treated effluent samples collected in this study (Table 4-10) are consistent with those found in the literature of which a comprehensive selection is presented in Table 4-2. Bezafibrate and carbamazepine were more frequently detected in both the screened and settled sewage and treated effluent than the other compounds which is consistent with the findings reported by Jelic et al. (2011). This was not surprising considering the high annual prescription levels identified in Table 4-12 and considering the numerous reports from other studies such as those collated in Table 4-2. In addition, bezafibrate and carbamazepine are typically prescribed for long term usage to treat chronic conditions compared to those which are typically prescribed for short term treatment of bacterial infections (e.g. enterococci and *E.coli*). Therefore, it is expected that bezafibrate and carbamazepine will be more prevalent than antibiotics in wastewaters than antibiotics.

Despite high reported prescription quantities (12 tonnes a^{-1}), clarithromycin could not be detected in the screened sewage, and was detected only once in settled sewage. Ciprofloxacin was detected in the wastewater but less frequently than bezafibrate and carbamazepine. Although higher concentrations of ciprofloxacin compared to the other compounds were observed in the screened and settled sewage this was not the case in the treated effluent (Table 4-10).

In a long term study of wastewater influents and effluents by Gros et al. (2010b), bezafibrate and carbamazepine were detected more frequently than ciprofloxacin which is

consistent with the findings in this work. However in the same study, clarithromycin was detected in 100 % of wastewater samples which contradicts the findings given in Table 4-10. This is possibly due to the limitations of the analytical method used in this study. The method limit of detection determined for clarithromycin in wastewaters (see Table 4-9) is greater than the typical wastewater concentrations reported by Gros et al. (2010b).

4.4.1.3 Comparison of predicted and measured influent concentrations of target pharmaceuticals

There are many factors that can influence the concentrations of pharmaceuticals present in wastewaters. These include population characteristics, wastewater treatment plant age and design, wastewater treatment process employed and seasonal changes (higher pharmaceutical loads have been encountered in winter compared to summer) (Loraine et al., 2005, Gros et al., 2010). Pharmaceutical consumption is considered to be a critical influencing parameter. In this study, prescription data was used to indicate the levels of the selected pharmaceuticals in sewage entering the monitored WWTP. Based on the prescription data, the highest predicted concentration for the wastewater influent ($PEC_{influent}$) was carbamazepine (~ 7900 ng/L) followed by clarithromycin (~ 2300 ng/L), bezafibrate (~ 1400 ng/L) and ciprofloxacin (~ 1300 ng/L).

Although prescribed in the largest quantities, carbamazepine was found in the screened sewage at levels much lower (369 – 1460 ng/L) than those predicted (~ 7900 ng/L). In addition, carbamazepine was present at lower concentrations than bezafibrate and ciprofloxacin in the screened and settled sewage (Figure 4-6). This is consistent with reports

by Collado et al. (2014) who found elevated levels of bezafibrate and ciprofloxacin compared to carbamazepine in wastewater influent (see Table 4-2). In addition, Gros et al. (2010b) found elevated levels of fluoroquinolones (including ciprofloxacin) and bezafibrate compared to carbamazepine, although the actual concentrations detected were not reported.

The lower than expected levels of carbamazepine detected in the screened sewage is probably due to the low proportion of carbamazepine excreted as the original compound. Following ingestion, pharmaceutical compounds will be excreted either as the original compound or as free or conjugated metabolites (Garcia-Ac et al., 2009). Not all pharmaceuticals are metabolised to the same extent and the proportion excreted as the original compound depends on the pharmacokinetics of the drug. Carbamazepine undergoes extensive metabolism in the human body and less than 10 % is excreted as the original compound. More than 30 human metabolites have been identified (Maggs et al., 1997) and consequently some have been identified in wastewaters including 10, 11 –dihydro- 10, 11 epoxycarbamazepine, 10, 11 –dihydro- 10, 11 dihydroxycarbamazepine, 2-hydroxycarbamazepine, 3 hydroxycarbamazepine, acridone and acridine (Leclercq et al., 2009). Furthermore, 10, 11 –dihydro- 10, 11 –dihydroxycarbamazepine has been identified in wastewaters at levels greater than the parent compound (Bahlmann et al., 2014; Miao et al., 2003; Leclercq et al., 2009).

Taking into account the proportion of the drug excreted in the original form (excretion factor), the predicted level ($PEC_{influent\ unchanged}$) for carbamazepine was more consistent with

those measured in the screened sewage (see Table 4-13). Compared to carbamazepine, a larger proportion of bezafibrate and ciprofloxacin are excreted in the unchanged form and therefore, the predicted influent levels excreted unchanged ($PEC_{influent\ unchanged}$) were within the range of concentrations detected in the screened sewage. There were no reports found on the occurrence of bezafibrate and ciprofloxacin human metabolites in wastewaters during the literature search for this study. The predicted level for clarithromycin was at a similar level to the corresponding method limit of detection (MLOD) and therefore it was not surprising that this compound was not detected in the screened sewage.

The concentrations of the target pharmaceuticals in the screened sewage varied between individual samples and will have been influenced by catchment characteristics including diurnal (general habits of individuals) and daily variations (e.g. working days vs weekends) (Ternes et al., 2008). Researchers have reported seasonal (Birosova et al., 2014; Li et al., 2011; McArdell et al., 2003) and temporal (Musolff et al., 2009) differences in pharmaceutical WWTP influent levels. Plosz et al. (2010) found concentrations of antibiotics in WWTP influent reduce throughout the day with maximum levels identified in the morning. The fluctuations in concentrations due to catchment characteristic will not have been captured using the grab sampling technique employed in this study. To evaluate pharmaceutical loads or mass fluxes in WWTPs, composite sampling techniques have been recommended (Ort et al., 2010). Analytical errors may have also contributed to the variations of pharmaceutical concentrations in the screened sewage and in the other samples. In this study, the standard addition technique was used as a method of quantification and to compensate for sample losses due to sample extraction. This

procedure is considered an accurate method for quantifying compounds in complex matrices (Ternes et al., 2008). However, the estimated errors (standard deviations) associated with quantifying the target compounds by extrapolation in wastewater and surface water samples were, as expected, sometimes quite large (Table 4-7).

4.4.1.4 Removal of target pharmaceuticals during the wastewater treatment processes

The removal of pharmaceuticals throughout the wastewater treatment process is complex but two important mechanisms are sorption and biodegradation (as presented in Section 2.2.1.2). The removal efficiencies of pharmaceuticals will vary depending on their tendency to sorb to sludge material or to the extent of biodegradation by micro-organisms.

In this study, high overall removal rates from wastewater were observed for ciprofloxacin (94.3 ± 0.5 %) whilst low removal rates were found for carbamazepine (22.1 ± 11.9 %). Since neither of these compounds is readily biodegraded (Zhang et al., 2008; Kümmerer et al., 2000; Al-Ahmad et al., 1999), sorption will be the predominant removal mechanism. In part, their different physico-chemical properties influence their sorption to wastewater solids together with the ambient pH. At neutral pH, ciprofloxacin mainly exists as a zwitterion carrying a positive charge from the protonation of a secondary amine of the piperazinyl moiety (see Section 4.1.1). Compounds that exhibit a positive charge are likely to interact with the negatively charged surface of microorganisms of activated sludge material (Ternes et al., 2004). The high activated sludge solid-water distribution coefficient (K_d) value determined for ciprofloxacin (2600 L/kg) confirms the affinity of this compound for activated sludge (Golet et al., 2003).

Looking at the wastewater treatment process more closely, it was observed that a high proportion of ciprofloxacin was removed (77.1 ± 24.6 %) during activated sludge treatment (see Table 4-11) which is consistent with the activated sludge K_d value determined by Golet et al., (2003) and with the activated sludge removal rates observed by Zorita et al., (2009). However, for compounds containing functional groups that can be protonated or deprotonated, the ambient pH is an important parameter influencing the level of sorption (Polesel et al., 2014). The pH values determined for the wastewater samples in this study were within the 6.8 – 7.1 range and may account for the different proportions of ciprofloxacin removed during activated sludge treatment (40.9 – 95.8 % as shown in Figure 4-7). As the ambient pH changes, the proportion of ciprofloxacin that exists in the zwitterionic form will change and consequently the capacity to sorb. The maximum sorption capacity for ciprofloxacin has been observed at its isoelectric point (pH 7.4) in sorption experiments carried out by Polesel et al., (2014).

A lower proportion of ciprofloxacin was removed during primary sedimentation (15.1 ± 62.4 %). This is expected as the proportion of microorganisms is lower in primary sludge compared to activated sludge and the expected impact is consistent with the lower K_d value determined for primary sludge (260 L/kg) by (Golet et al., 2003). The removal by sorption processes in wastewater treatment plants is considered negligible for compounds with solid-water distribution coefficients < 300 L/Kg (see Chapter 2, Section 2.2.1.3)

Carbamazepine is a neutral molecule at neutral pH and therefore sorption to wastewater solids will be mainly through hydrophobic interactions. However, the octanol-water

coefficient for carbamazepine is low (pKow= 2.45, see Table 4-5) and uncharged chemicals with log Kow < 2.5 are predicted to show a low sorption potential (Golet et al., 2003). This is agreeable with both the sludge-water adsorption coefficient (K_d) determined for primary sludge material (20 L/kg) and for activated sludge material (1.2 L/Kg) suggesting a low level of sorption to wastewater particulate matter (Ternes et al., 2004). Therefore, the low overall removal of carbamazepine through the wastewater treatment process is explained by its physicochemical properties and is consistent with observations found by Jelic et al. (2011).

A closer inspection of the removal of carbamazepine at different stages within the wastewater treatment process revealed that primary sedimentation processes provided the main contribution to the removal of carbamazepine (20.0 ± 29.7 %) with an average negative removal (-4.7 ± 52.9 %) being observed for activated sludge treatment (see Table 4-11). Similar results were reported by Vieno et al. (2006); Clara et al. (2004) and Gros et al. (2010) and attributed to the desorption of carbamazepine from wastewater solids. Carbamazepine absorbs to wastewater solids through hydrophobic interactions. However, hydrophobic components of wastewater solids are degraded during biological treatment thus releasing the compound back to the aqueous phase (Ternes et al., 2008). Furthermore, it has been reported that carbamazepine levels may increase due to the deconjugation of carbamazepine metabolites in the activated sludge tank, thus releasing the parent compound (Vieno et al., 2007). The metabolites and transformation products of the target pharmaceuticals were not investigated in this study.

In the removal of bezafibrate, the activated sludge treatment process substantially contributed to the high overall removal rate (89.7%) and this is consistent with removal values reported elsewhere (Radjenovic et al., 2009; Castiglioni et al., 2004). Sorption during the activated sludge treatment process is not considered to be an important removal mechanism for bezafibrate. This is because at neutral pH it is in the anionic form and will therefore not interact with the negatively charged surfaces of microorganisms present in activated sludge. However, Quintana et al. (2005) and Ternes (1998) reported that biodegradation is an important removal mechanism for bezafibrate during the activated sludge process.

The removal of clarithromycin during the primary sedimentation process could not be assessed as this compound was not detected in the screened sewage above the method limits of quantification. However, it has been reported by Göbel et al. (2007) that primary sedimentation does not significantly reduce the levels of clarithromycin. Joss et al. (2006) demonstrated that biodegradation is not an important removal mechanism for clarithromycin in the activated sludge processes. Therefore the removal of 59.5 % of clarithromycin following activated sludge treatment suggests sorption is an important removal mechanism. In addition, clarithromycin was expected to sorb onto the negatively charged surface of activated sludge because it carries a positive charge (through the protonation of the tertiary amino group) at neutral pH. However, the moderate monitored removal of clarithromycin contradicts a report by Göbel et al. (2005) in which < 5% was removed during activated sludge treatment. In addition, the solid-water distribution coefficient (260 L/Kg) determined by Golet et al. (2003) for clarithromycin suggests

negligible sorption onto activated sludge material. However, the sorption of compounds can vary due to variations in the sewage composition (Ternes et al., 2004). In addition, the removal of clarithromycin (59.5 %) during the activated sludge process is only based on one settled sludge measurement and therefore further measurements would be required for a better assessment of the fate of clarithromycin during wastewater treatment processes. The overall reduction of clarithromycin could not be calculated, as clarithromycin was not detected in the screened sewage.

The removal of the target pharmaceuticals (in particular carbamazepine) was found to be variable in this study with both negative and positive removal efficiencies observed (Figure 4-7). Differences in the wastewater treatment plant operating conditions (e.g. the hydraulic retention time (HRT) and sludge age (SRT)) may account for some of the variability (Verlicchi et al., 2014b). Clara et al., (2005) found that bezafibrate removal was more efficient with a sludge age > 10 days presumably because this equates to a larger microbial diversity and consequently to more varied potential biodegradation pathways. However, Gros et al., (2010) found changes in HRT did not affect the removal of carbamazepine and Vieno et al. (2007) reported no obvious correlation between the proportion of carbamazepine and ciprofloxacin removed and SRT.

The variability in the observed removal efficiencies could be due to the limitations of the grab sampling technique used in this study. Grab samples only provide an instantaneous measurement of the target pharmaceutical concentration. Therefore this technique will not compensate for the short and long term fluctuations that may occur in wastewater

treatment plants due to changes in hydraulic retention time and sludge retention time (Ternes et al., 2008). Some studies have used composite sampling techniques over 24 hrs (Golet et al., 2002) to account for such variations. However, despite using composite sampling techniques, Gros et al. (2010b) also reported both positive and negative removal efficiencies for bezafibrate, carbamazepine and ciprofloxacin consistent with those found in this study.

4.4.1.5 Occurrence of target pharmaceuticals in surface water

Although at lower concentrations than those observed for sewage and treated effluent samples, bezafibrate, carbamazepine, ciprofloxacin and clarithromycin were detected in the surface water down-stream of the treated effluent discharge point of the WWTP. In the treated effluent, carbamazepine was present in higher concentrations compared to the other selected pharmaceuticals and correspondingly it was also present at higher concentrations in the surface water (Figure 4-6). This perhaps reflects the resistance of carbamazepine to biodegradation and sorption processes. These findings are consistent with those reported by Fernandez et al. (2010), Daneshvar et al. (2010) and Petrovic et al. (2014) who also found elevated levels of carbamazepine compared to other pharmaceutical compounds in surface waters.

Both bezafibrate and carbamazepine were detected in the surface water up-stream relative to the treated effluent discharge point. The occurrence of pharmaceuticals in the surface water up-stream of the WWTP samples indicates there are alternative sources of pharmaceuticals further up-stream. This is probably due to a number of misconnections of

the ageing sewer infrastructure and discharges of sewage from combined sewerage overflows to the surface water within this area (Thames Water, 2014).

Where detected, the pharmaceutical levels were typically higher downstream compared to upstream of the WWTP treated effluent discharge point (Figure 4-8). This demonstrates that pharmaceutical compounds are incompletely removed during the wastewater treatment process and that surface waters are vulnerable to pharmaceutical contamination from point sources. Ciprofloxacin and clarithromycin were only detected down-stream of the WWTP discharge point. This perhaps indicates that bezafibrate and carbamazepine are more ubiquitous and persistent than ciprofloxacin and clarithromycin in surface water. Furthermore, it suggests the constant release of pharmaceutical contamination to surface waters within WWTP treated effluent discharges. It is possible that ciprofloxacin and clarithromycin were present in upstream samples at levels below the method detection limits (25 and 10 ng/L respectively) and therefore not detected. In addition, the concentrations of the selected pharmaceuticals in surface water samples may have been underestimated as a consequence of filtration prior to extraction, removing any suspended solid bound pharmaceuticals. Silva et al., (2011) observed bezafibrate, carbamazepine and clarithromycin in the suspended solid samples of various river water samples but not always in the corresponding aqueous phase. Furthermore, 30 % of the 43 pharmaceuticals investigated were found to be predominantly associated with suspended solids compared to the aqueous phase.

The measured target pharmaceuticals (Table 4-10) in the surface water are consistent with those reported by other researchers (Table 4-3) for river waters receiving treated effluent discharges. The data in Table 4-3 show that although present in variable concentrations bezafibrate, carbamazepine, ciprofloxacin and clarithromycin, if detected in surface waters are generally present at ng/L concentrations. Surprisingly, there are reports that these compounds have been detected at concentrations in excess of 1 μg/L (Valcarcel et al., 2011; Watkinson et al., 2009; Feitosa-Felizzola et al., 2009).

Pharmaceutical consumption levels and WWTP pharmaceutical removal efficiencies will initially influence the levels of these compounds discharged to receiving waters but subsequently further sorption, and photodegradation processes may take place in the surface water (Batt et al., 2006). Photodegradation may be an important removal process for some pharmaceuticals in surface waters. However, Cardoza et al., (2005) found the presence of particulate organic carbon dramatically reduced the photodegradation of ciprofloxacin. Similarly, the presence of humic acids reduced the photodegradation of carbamazepine in studies conducted by Andreozzi et al., (2002) and photodegradation was found to have a limited impact on clarithromycin in surface water (Vione et al., 2009). In the current study, high levels of suspended solids were measured in the surface water (see Table 4-4) and therefore may have limited UV light penetrating the surface limiting removal by photodegradation.

Pharmaceutical compounds with higher soil/water organic carbon sorption coefficients (K_{oc}) may sorb to surface water particulates and therefore be removed from the surface water.

^{14}C-ciprofloxacin studies by Cardoza et al. (2005) confirmed ciprofloxacin to be significantly adsorbed onto aquatic particulate organic carbon (K_{oc} values of 13,900 to 20,500 L/kg at neutral pH). It may be that sorption processes partially explain why ciprofloxacin was infrequently detected in the surface water samples in this study (see Table 4-10). When detected, high levels of ciprofloxacin may have been released from the WWTP in a non-sorbed state or perhaps desorption from particles and solids occurred in the surface water. Desorption may occur due to changes in the ambient pH. pH values can greatly affect the behaviour of ionisable compounds (Verlicchi et al., 2014b). For example, Polesel et al. (2014) found that ciprofloxacin had reduced sorption capacity at a pH value of 6.3 (K_d = 366 L/kg) and a pH value of 8 (K_d =371 L/Kg) compared to a pH value of 7.4 (K_d = 1225 L/Kg). The pH values determined for the surface water down-stream of the WWTP treated effluent discharge point ranged between 7.2 and 8.3 (mean ± standard deviation = 7.8 ± 0.4) and therefore corresponded to levels at which the sorption capacity of ciprofloxacin could have been expected to decrease. In contrast to ciprofloxacin, carbamazepine is expected to sorb much less to surface water particulates based on the experimentally derived K_{oc} value of 521 L/Kg (Drillia et al., 2005) and its low octanol-water coefficient (282) enabling it to be detected in the majority of surface samples analysed in this study (Table 4-10).

Where detected, the target pharmaceuticals have been detected in surface waters up- and down-stream of the WWTP discharge point at levels greater than the action limit (10 ng/L) given by regulatory guidelines for the environmental risk assessment of new pharmaceuticals to market (European Medicines Agency 2006). An array of tests including the algal growth inhibition test (OECD 201) and *Daphnia sp*. reproduction test (OECD 211)

have been recommended for pharmaceutical compounds with predicted surface water concentrations that exceed this action limit (Chapter 2, Section 2.5.4). However, the tests are performed on a small selection of organisms and therefore the effects on specific organisms may be missed. In addition, there is no recommendation for tailored tests to investigate the effects that may be specific to certain groups of pharmaceuticals. The effects of antibiotics in aquatic ecosystems and the potential to select for antibiotic resistant bacteria is an example. Most importantly, bezafibrate, carbamazepine, ciprofloxacin and clarithromycin are exempt from environmental risk assessment as the current legislation (European Commission, 2001) only applies to new medicines to the market (Section 2.5.4) and therefore information on the fate and effects of these compounds is not comprehensive. This is of concern as there are reports indicating the potential risk of these selected compounds in aquatic ecosystems (Martins et al., 2012; Isidori et al., 2005; Halling-Sorensen et al., 2000).

4.5 Summary

Using solid phase extraction combined with LC-MS, bezafibrate, carbamazepine, ciprofloxacin and clarithromycin have been detected in the wastewaters of a large urban wastewater treatment plant and in the surface waters receiving the WWTP discharged treated effluent. Method detection limits ranged between 5 and 500 ng/L for surface waters and screened sewage respectively. The analysis of prescription data has indicated the high quantities of four pharmaceuticals (bezafibrate, carbamazepine, ciprofloxacin and clarithromycin) prescribed per year that could ultimately arrive at wastewater treatment

plants following ingestion and excretion. The analysis of wastewaters and samples collected both up- and down-stream of the discharged effluent from a large wastewater treatment plant show that these compounds are incompletely eliminated. Although the percentage removed during wastewater treatment depends on a number of factors including the type of treatment and the population characteristics, sorption is shown to be an important removal process particularly for ciprofloxacin. A comparison of pharmaceutical concentrations up- and down-stream of the discharged effluent suggests receiving waters are vulnerable to pharmaceutical contamination from point sources.

5 Antibiotic resistance patterns of *Escherichia coli* and enterococci in an urban environment

5.1 Introduction

Antibiotic resistant bacteria and antibiotic residues are present in wastewater and it is a concern that wastewater treatment plants provide a hotspot for the dissemination of antibiotic resistance which could ultimately impact surface waters receiving treated wastewater effluent discharges.

Few studies have investigated antibiotic resistance amongst bacteria in environmental waters such as wastewater treatment plants and surface waters and typically in these studies bacteria are assessed using qualitative antibiotic susceptibility tests (e.g. disc diffusion) that define bacteria as being either resistant or susceptible according to clinical breakpoint values (Servais et al., 2009; Faria et al., 2009). However, clinical breakpoint values (CBPs) may differ between different antibiotic susceptibility testing methods, can vary internationally and may be set at different antibiotic levels in animals and humans (Kahlmeter et al., 2003). This lack of internationally accepted harmonised breakpoints makes it difficult to interpret and compare the resistance levels reported by different studies. In addition, clinical breakpoint values are primarily established for guidance on therapy and distinguish between treatable and non-treatable bacteria influenced by pharmacodynamic and pharmakinetics data. They are not always appropriate for subtly detecting emerging phenotypic resistance. Quantitative antibiotic susceptibility methods

result in an antibiotic concentration that can inhibit visible growth of micro-organisms (minimum inhibitory concentration) and can be interpreted using epidemiological cut off values (ECOffs) or clinical breakpoint values if required. Epidemiological cut off values (ECOffs) have been established by The European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2010) and are used for the detection of bacteria with acquired resistance mechanisms and for the sensitive detection of emerging resistance. The cut off value will remain the same despite changes in antibiotic therapy in humans and animals (Kahlmeter et al., 2003). Antibiotic susceptibility testing and interpretative breakpoints and epidemiological cut off values have been described in more detail in Chapter 3, Section 3.4.

In this chapter, the presence of bacteria indicative of human faecal contamination and their respective antibiotic resistant sub populations in environmental waters (settled sewage, final treated effluent and surface water) are investigated. Water samples are collected from the settled sewage and final treated effluent associated with a large urban wastewater treatment plant and in surface waters both up- and down-stream of the discharge point of the final effluent, in which antibiotics have previously been detected (presented in Chapter 4). Quantitative antibiotic susceptibility tests are interpreted using harmonised clinical breakpoint values and epidemiological cut off values defined by EUCAST (2012). It was decided to use both harmonised clinical breakpoint and epidemiological cut off values as currently there are no accepted standardised procedures to assess the transfer of antibiotic resistance in environmental waters. In addition, harmonised clinical breakpoint values are considered when epidemiological cut off values for certain bacteria/antibiotic combinations have yet to be determined.

5.1.1 Selection of bacteria

Coliform bacteria, enterococci, staphylococci and *Pseudomonas aeruginosa* are all potentially pathogenic bacteria and are common causes of clinical infections (Health Protection Agency. 2007). They are all associated with the gastrointestinal tract of humans and animals and consequently are detected in sewage. Their use as microbial indicators of water quality is presented in Chapter 3, Section 3.1. Whilst in the host, coliforms, enterococci, staphylococci and *Pseudomonas aeruginosa* are exposed to a variety of medical and veterinary antibiotic treatments and consequently can become resistant to the antibiotics used against them (Section 3.4). Therefore coliform, enterococci, staphylococci and *Pseudomonas aeruginosa* bacteria were selected for consideration as representative bacterial groups to study the transfer of antibiotic resistance from wastewater to receiving surface waters. In addition, it was decided to perform a heterotrophic count on all water samples as an indication of the levels of culturable bacteria. However, preliminary studies revealed that the detection and identification (to species level) of staphylococci (including *Staphylococcus aureus*) and pseudomonas *(*including *Pseudomonas aeruginosa)* from environmental waters was difficult and therefore subsequent antibiotic susceptibility tests were not performed on these organisms (Sections 5.3.3 and 5.3.4).

Antibiotic resistance can vary between species of a bacterial group (e.g. coliforms) or genera (e.g. enterococci). For example, within the coliform group there are different genera including *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Serratia* and *Yersinia* and antibiotic resistance in some maybe more predominant than in others. Whilst within the

Enterococci genera, *Enterococcus faecium* are considerably more resistant to penicillins than *Enterococcus faecalis* bacteria. In addition, *Enterococcus casseliflavus* and *Enterococcus gallinarum* inherently display a low level resistance to vancomycin whereas other species of the group do not (Eliopoulos, 2007). Therefore, it was decided that identification to species level was necessary to help understand the antibiotic resistance patterns observed in the collected wastewater and surface water samples. The final selection of bacterial species for study was based on the efficiency of bacteriological analysis methods and the frequency with which individual species were detected in the collected water samples (Section 5.3.2). The bacterial species selected were *Escherichia coli* and *Enterococcus faecium*.

5.1.2 Selection of antibiotics for susceptibility testing

Amoxicillin and ciprofloxacin are important for the treatment of both Gram negative and gram positive bacterial infections and correspondingly are prescribed in large quantities in England (~ 228 and 7 tonnes for amoxicillin and ciprofloxacin respectively per year). Approximately 80 % and 50 % of a dose of amoxicillin and ciprofloxacin respectively are excreted in the original form and potentially transferred to wastewater treatment plants. Ciprofloxacin has been detected in wastewater and receiving surface waters in this study (presented in Chapter 4).

The surveillance of penicillin resistance (including amoxicillin) in *E.faecium* and *E.coli* in clinical settings within the UK has indicated high rates of resistance. Surveillance has shown that penicillin resistance in *E.faecium* has increased from 77.6 % to 93.1 % between 2005 and 2012 (European Centre for Disease Prevention and Control - Antimicrobial resistance

interactive database (EARS-net), 2014). Whilst, the levels of penicillin resistance in *E.coli* was reported to be 62.7 % (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2013b).

There is no mandatory surveillance of fluoroquinolone resistance in *E.faecium* in the UK, but there are studies that report resistance to fluoroquinolones is widespread in the enterococci genus (Eliopoulos, 2007). Surveillance of *E.coli* has demonstrated that fluoroquinolone resistance levels reached 16.6 % in the UK in 2012 (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2013c). In addition, ciprofloxacin has been classified by the World Health Organization (2011) as a critically important antibiotic. The classification describes antibiotics that are the sole treatment for serious human infections and for antibiotics that are used to treat infections caused by bacteria from non-human sources (World Health Organization, 2011). Due to their importance in human medicine and the potential impact on environmental waters, both amoxicillin and ciprofloxacin were chosen for this work.

Clarithromycin is a macrolide antibiotic prescribed in high quantities each year (~ 17 tonnes a.i in England) and a substantial proportion (~ 25 %) is excreted in the active form (Ternes et al., 2008). Furthermore, clarithromycin has been detected in wastewaters and receiving surface waters (discussed in Chapter 4) and therefore was selected for further investigation. Macrolide antibiotics (including clarithromycin) constitute an important alternative therapy for the treatment of insidious enterococci infections (Portillo et al., 2000). However, Gram negative bacteria are intrinsically resistant to macrolides due to their outer membrane

(Chapter 3, Section 3.3.5.3). Therefore clarithromycin susceptibility studies were only carried out on *E.faecium* and not on *E.coli*.

The surveillance of vancomycin resistance in enterococci species is mandatory in clinical settings due to the importance of vancomycin therapy for enterococci infections. Clinical surveillance of vancomycin resistance in *E.faecium* have shown that resistance rates have reduced from 33.0 % (2005) to 13.3 % (2012) in the UK (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2014b). In addition, the prescription quantities of vancomycin are very low (0.03 tonnes a.i, in England) and there are very few reports of the detection of vancomycin in environmental waters (Chapter 4, Section 4.1.1). Vancomycin was selected for this work as a contrast to amoxicillin, ciprofloxacin and clarithromycin which are prescribed in high quantities in England and because of its importance in the treatment of methicillin resistant Staphylococcus aureus (Kos et al., 2012). Vancomycin resistance levels in *E.coli* were not investigated because of their intrinsic resistance to this antibiotic.

Cefpodoxime is considered a critically important antibiotic because it is one of a small number of Cephalosporins (3rd and 4th generation) that can be used to treat bacterial meningitis and diseases due to *Salmonella* in children (World Health Organization, 2011). enterococci species are intrinsically resistant to cephalosporins (e.g. cefpodoxime), (European Antimicrobial Resistance Surveillance Network, 2011). However, clinical surveillance has shown that resistance to 3rd generation cephalosporins (including cefpodoxime) in *E.coli* has increased (2001 – 2012) from 1.2 – 13.1 % in the UK (European

Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2013a). This is despite the low quantities of cefpodoxime prescribed each year (0.002 tonnes a.i, in England). Although cephalosporins are rarely detected in environmental waters (see Chapter 4, Section 4.1.1), cefpodoxime was selected for susceptibility testing in *E.coli* as a contrast to amoxicillin and ciprofloxacin.

5.2 Materials and methods for bacterial analysis

5.2.1 Method overview

In this chapter, the detection and enumeration of coliform bacteria, *Escherichia coli*, enterococci, staphylococci and pseudomonas in wastewater and surface water samples are described (presented in Section 5.2.5). A membrane filtration method was selected as it is a recognised bacteriological method for water quality monitoring (see Chapter 3, Section 3.2). Non-target bacteria can grow on the specific growth media used in membrane filtration and therefore the bacteria detected on the membrane filters were considered presumptive. A variety of confirmation tests (presented in Section 5.2.5.4) recommended by the Environment Agency (2000) were carried out on a proportion of the presumptive bacteria to confirm that they belonged to the target group (e.g. coliforms) or genera (e.g. enterococci). In addition, the effectiveness of a chromogenic agar to differentiate between *E.faecium* from other presumptive enterococci bacteria was assessed as a confirmation test.

Further testing was required to identify the detected bacteria to species level. Initially the identification of coliform bacteria, *E.coli*, enterococci, staphylococci and pseudomonas were

performed using commercial biochemical kits. However during the study an opportunity to use matrix assisted laser desorption-time of flight- mass spectrometry (MALDI-TOF-MS) for the identification (to species level) of isolated bacteria became available.

From the coliform and enterococci group, *E.coli* and *E.faecium* were selected for subsequent antibiotic susceptibility testing because they were frequently and easily detected (see Sections 5.3.2.1 and 5.3.2.5). *E.coli* (n = 229) and *E.faecium* (n = 129) detected in wastewater and surface water samples were isolated and processed for identification to species levels using MALDI-TOF-MS analysis and antibiotic susceptibility testing using antibiotic gradient strips. The principles and background for the methods used in this chapter are outlined in Chapter 3.

5.2.2 Study area

Full details of the study site have been given in Chapter 4, Section 4.2.2

5.2.3 Sample collection

Samples of settled sewage and final treated effluent were collected on five occasions, between July 2011 and February 2012, from an urban wastewater treatment plant employing activated sludge. Samples were also taken from surface waters up- and down-stream of the effluent discharge point (see Chapter 4, Section 4.2.2). Samples were collected simultaneously to the collection of samples for chemical analysis described in Chapter 4. Duplicate samples were collected in 500 mL sterile (Gamma radiated) bottles

(Sterilin, Ltd, UK) and stored in a cool box (with frozen ice packs) during transport to the laboratory. All samples were processed within 4 h of collection.

5.2.4 Media and reagents

Brilliance *E.coli* /coliform selective agar (BO1014M), Slanetz and Bartley agar (CM0377), Bile aesculin agar (CM0888), R2A agar (CM0906), Mannitol salt agar (CM0085), Pseudomonas base agar (CM0559) and *Pseudomonas aeruginosa* selective supplement (SR0102) which contains nalidixic acid and cetrimide and Mueller Hinton agar (CM0337), tryptone water (CM0087), Gram staining set (R40080 - contains crystal violet, iodine and safranin) and Microbat oxidase detection strips (MB0266) were purchased from Oxoid Ltd. Sodium azide (99 % SpeciFied) and 6 % hydrogen peroxide solution (CertiFied) were purchased from Fisher Scientific (UK). All media were prepared according to the instructions outlined by the manufacturer. API 20E (for Enterobacteriaceae), API 20 Strep (for the identification of enterococci), API Staph (to identify staphylococci) and API NE (for the identification of non-enteric Gram negative rods) identification kits and reagents (catalogue references: 20100, 20600, 20500 and 70050 respectively) and Etest® antibiotic gradient strips for ciprofloxacin (0.002-32 µg/mL), amoxicillin (0.016-256 µg/mL), clarithromycin (0.016-256 µg/mL), vancomycin (0.016-256 µg/mL) and cefpodoxime (0.016-256 µg/mL) were purchased from Biomerieux Ltd, UK (catalogue references 508618, 500918, 508618, 525518 and 505818 respectively).

5.2.5 Detection and enumeration methods

5.2.5.1 Membrane filtration method

The detection and enumeration of bacteria in the collected samples were carried out using the membrane filtration method. Samples were subjected to 10-fold serial dilutions with sterile water. 100 mL aliquots of the diluted samples were filtered (in duplicate) using 47 mm cellulose nitrate membrane filters (0.45 µm) and incubated on different culture media (as summarised in Table 5-1).

Table 5-1: Culture media used for the specific detection of target indicator bacteria

Target bacteria	Media	Incubation	[a] Results
E.coli/ coliforms	*E.coli*/coliform chromogenic agar	37 °C for 24 h	coliforms = purple colonies *E.coli* = pink colonies
Enterococci	Slanetz and Bartley agar	37 °C for 4 h & 44 °C for 40 h	*Enterococci* = maroon colonies
Staphylococci - including *Staphylococcus aureus*	Mannitol salt agar with 0.005 % sodium azide	30 °C for 24 h	Staphylococci = pink colonies *Staphylococcus aureus* = cream colonies
Pseudomonas -including *Pseudomonas aeruginosa*	Pseudomonas base agar with naladixic acid and cetrimide	35°C for 24 and 48 h	Pseudomonas = cream colonies *Pseudomonas* aeruginosa blue/green or red/brown colonies

[a] results are considered presumptive. Selective, differential and indicative properties of the growth media are given in Chapter 3, Section 3.2.

Blank (negative) controls of 100 mL sterile water were processed in the same manner as the samples. The enumeration of target bacteria were only carried out for plates displaying approximately 10 – 100 colonies.

5.2.5.2 Spread plate method

Heterotrophic bacteria counts were performed on the samples using the spread plate method following guidelines produced by the Environment Agency (2007). 0.1 mL aliquots of diluted samples (diluted by a 10-fold serial dilution with sterile water) were spread on R2A agar (low nutrient agar) in 90 mm petri dishes using sterile plastic bent rods in duplicate. For each sample one plate was incubated at 30 ± 5 °C for three days and the other at room temperature (21 ± 5 °C) for 7 days. A blank control was also processed (0.1 mL sterile water) to check for no growth.

5.2.5.3 Isolation and storage of isolates

Presumptive bacteria of interest were purified by sub culturing using nutrient agar. Working stocks of each isolate were stored on nutrient agar slants at 4° C and preserved in nutrient broth with 20 % glycerol (stored at -80 ° C) until further analysis.

5.2.5.4 Methods to confirm presumptive isolates

From each specific growth medium, a proportion of the presumptive colonies were isolated from plates, displaying growth of approximately 10-100 colonies for subsequent confirmation tests. The tests used are summarised in (Table 5-2). The enumeration of target

bacteria were adjusted for the proportion of isolates that were confirmed as belonging to the target group as identified by the Environment Agency (2000).

Table 5-2: Confirmation tests used for presumptive bacteria

Target	Test	Test details	Expected results
E.coli coliforms	indole test	Tryptone water was inoculated with colonies and incubated at 44 °C for 24 h. Two drops of Kovac's reagent was then added	***E.coli*** = red colouration with Kovac's reagent **Coliforms** = no change
E.coli Coliforms *Pseudomonas*	Oxidase test	Colonies were applied on to oxidase detection strips impregnated with NNN'N' tetramethyl -p- phenylene-diamine dihydrochloride	***E.coli*** = no change **Coliforms** = no change **Pseudomonas** = purple coloured formed in 5 s
Enterococci	aesculin hydrolysis	Colonies were streaked on to bile aesculin agar and incubated at 44 °C for 24 h	**Enterococci** = blackening of the agar
Enterococci	growth in 6.5 % sodium chloride (NaCl)	Colonies were inoculated into nutrient broth containing 6.5 % (weight/volume) NaCl and incubated at 44 °C for 24 h	**Enterococci** = If the broth is turbid following incubation
Staphylococci	Gram stain	Colonies were applied to a microscope slide. Crystal violet dye is added, left for 1 min and then washed off. Gram's Iodine solution is added and washed off after 1 min. 95 % alcohol was added and washed off after 10 s. Safranin was added washed off after 30, ready for microscope analysis	**Staphylococci** = Gram Positive
Staphylococci	catalase	3 % hydrogen peroxide is added to colonies applied to microscope slides	**Staphylococci** = bubbles formed

More details on the confirmation tests are given in Chapter 3, Section 3.2.1.1.

5.2.5.5 Evaluation of growth media efficiency

Presumptive *E.coli* (50 isolates), coliform bacteria (30 isolates), enterococci (61 isolates) staphylococci (62 isolates) and pseudomonas (86 isolates) taken from wastewater and surface water samples and reference control strains (see Section 5.2.8) were identified to species level (by either phenotypic identification or a combination of phenotypic identification and MALDI-TOF-MS analysis). This was to evaluate the efficiency of the growth media used to detect the target bacteria and inhibit non-target bacteria. The growth media constituents used for the selective and differential detection of target bacteria are given in Chapter 3, Section 3.2.

5.2.5.6 Evaluation of a chromogenic agar to differentiate E.faecium from other Enterococci species

While enterococci are quite easily cultivated on Slanetz and Bartley media, the isolation and differentiation of a specific species such as *Enterococcus faecium* from mixed enterococci populations can be problematic. This is because different species of enterococci produce colonies of similar appearance on Slanetz and Bartley media. Therefore, the effectiveness of a chromogenic media (cephalextin arabinose agar) to differentiate between *E.faecium* and presumptive enterococci isolates was evaluated. The intention was to use the agar as a confirmation test to specifically detect *E.faecium* from a mixed enterococci population. The red coloured chromogenic media utilises a chromogenic substrate to specifically detect the presence of the enzyme β-glucosidase which is characteristic of enterococci. Cleaving the substrate produces blue presumptive enterococci colonies. *E.faecium* can be differentiated

from other enterococci species due to the presence of arabinose in the agar. Species such as *E.faecalis* does not ferment arabinose and therefore retains the blue colour. Conversely, *E.faecium* does ferment arabinose producing green coloured colonies. A yellow colouration to the medium is also produced upon fermentation of arabinose as the media also contains phenol red pH indicator. The agar is supplemented with aztreonam and cephalextin to inhibit Gram negative bacteria and Gram positive bacteria other than enterococci.

Presumptive enterococci isolates (n = 262) taken from surface and wastewaters and control reference strains (see Section 5.2.8 were sub-cultured onto the chromogenic media. Following incubation 153 green (presumptive *E.faecium*) and 109 blue (presumptive other Enterococci species) colonies were observed and identified using matrix assisted laser desorption- time of flight (MALDI-TOF-MS) analysis.

5.2.6 Identification methods

5.2.6.1 Phenotypic identification methods

Phenotypic identification to species level was performed using commercial biochemical standardised systems (API®, Biomerieux). There are different systems for *Enterobacteriaceae*, staphylococci, streptococci and Gram negative rods. Each API® system comprises of a strip holding a series of different diagnostic media contained in microtubes used to detect certain metabolic reactions specific to the group of bacteria in each system (Figure 5-1).

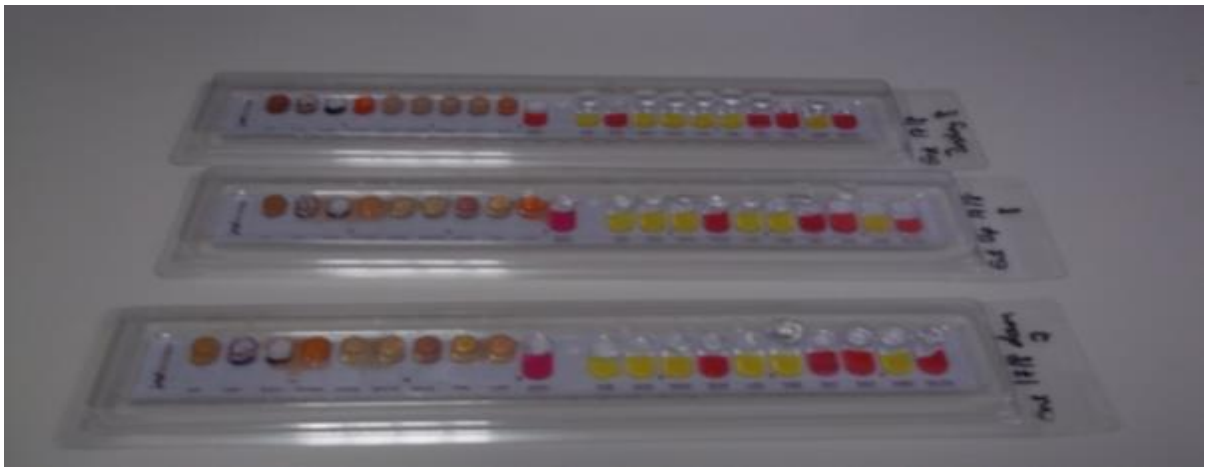


Figure 5-1: API 20 Strep strips used for the identification of presumed enterococci isolates

For isolates to be identified, suspensions were made from single well isolated young colonies suspended in 5 mL 0.85 % NaCl sterile solution. Bacterial suspensions were used to inoculate the dehydrated media on the relevant API strip and then incubated. The diagnostic media used in each system are shown in Appendices 1 to 4. The resulting changes to the media are used to create a biochemical profile of the tested isolate. For identification purposes the biochemical profile is compared to the profile of 600 species of bacteria in a database (apiweb™ software (v 1.2.1).

The proposed identification is supported by the calculation of two indices; the identification percentage (the frequency of the unknown biochemical profile occurring for the proposed species) and the T index (comparison of the unknown biochemical profile to the most typical profile for the proposed species). The level of identification produced by the software is categorised as either excellent (≥ 99.9 % id and ≥ 0.75 T index), very good (≥ 94.9 % id and ≥ 0.5 T index) or good (≥ 90.0 % id and ≥ 0.25 T index). Only isolate identifications categorised

as good, very good or excellent were used in this study. If the level of identification was < 90 %, the bacteria under test was considered to be unidentified by this approach.

5.2.6.2 MALDI-TOF-MS identification method

Initially, MALDI-TOF-MS analysis was not available for this study. Therefore, the identification of presumptive coliform bacteria, *E.coli* (see Section 5.3.2.1), staphylococci (see Section 5.3.2.2), pseudomonas (see Section 5.3.2.3) and enterococci (see Section 5.3.2.4) to species level was performed using phenotypic biochemical kits. When MALDI-TOF-MS became available, presumptive enterococci isolates were identified to species level using this technique. In addition, all isolates (presumptive *E.coli* and *E.faecium*) selected for subsequent antibiotic susceptibility testing were identified to species level using this technique to confirm species identity.

MALDI-TOF-MS identification of isolates was performed on fresh overnight cultures (Mueller Hinton agar) using a MALDI Biotyper (Bruker Daltonics, Billerica, MA) at the Department for Bioanalysis and Horizon Technologies, Public Health England (PHE). Samples for analysis were prepared using the ethanol/formic acid extraction method. A loop full of a fresh culture was homogenised in 300 µL deionised water in an Eppendorf tube. The mixture was vortexed and then 900 µL of ethanol was added. The mixture was mixed again and then centrifuged at 18,000 r.p.m for 2 min. The supernatant was decanted and the remaining pellet centrifuged again. The residual ethanol/deionised water was removed with a pipette and the pellet left to air dry for a further 2 min. Acetonitrile (50 µL) and 70 % formic acid (50 µL) were added to the pellet and centrifuged for 2 min. 1 µL of the supernatant was

transferred to a spot on a MALDI target (96 spot, polished steel plate). Once the spots had dried, 2 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics) matrix was applied on top of each spot and left to dry prior to MALDI-TOF-MS analysis.

Analytical methods were validated, optimised and calibrated by PHE staff scientists. Routine protein calibrations were performed using a bacterial test solution containing an extract of the strain *E.coli* DH5 alpha spiked with additional proteins to cover a mass range between 2 and 20 kDa. Protein calibrations were performed to optimise the laser intensity and to ensure the mass errors for the measured masses of the test solution proteins were within 300 parts per million (mass error/exact mass x 10^6) of the reference masses.

The MALDI Biotyper consists of a MicroFlex bench top MALDI mass spectrometer with a nitrogen laser (337 nm) operated in positive linear mode (voltage 20 Kv; mass range 2 – 20 kDa) controlled by FlexControl version 3.3. A mass spectrum of mainly ribosomal intrinsic proteins from each sample was obtained by averaging 40 pulsed shots acquired in automatic mode.

Identification of the microorganisms from the acquired mass spectra was achieved using MALDI Biotyper Realtime Classification software (version 3.1) which compares the acquired spectra to all entries in a database containing more than 3700 spectra entries representing approximately 319 genera and 2000 species. Unknown samples are given a score which is based on a matching algorithm to reference samples and reflects the level of identification obtained.

The score value defined by three components, the percentage of matches of peaks in the unknown spectrum compared to the total peaks in the reference spectrum (%), the percentage matches of the peaks in the reference spectra to the total peaks in the unknown spectrum (%) and the correlation of the intensities of the matched peaks (between 0 to 1). An example of how mass spectra are compared and interpreted is shown in Figure 5-2.

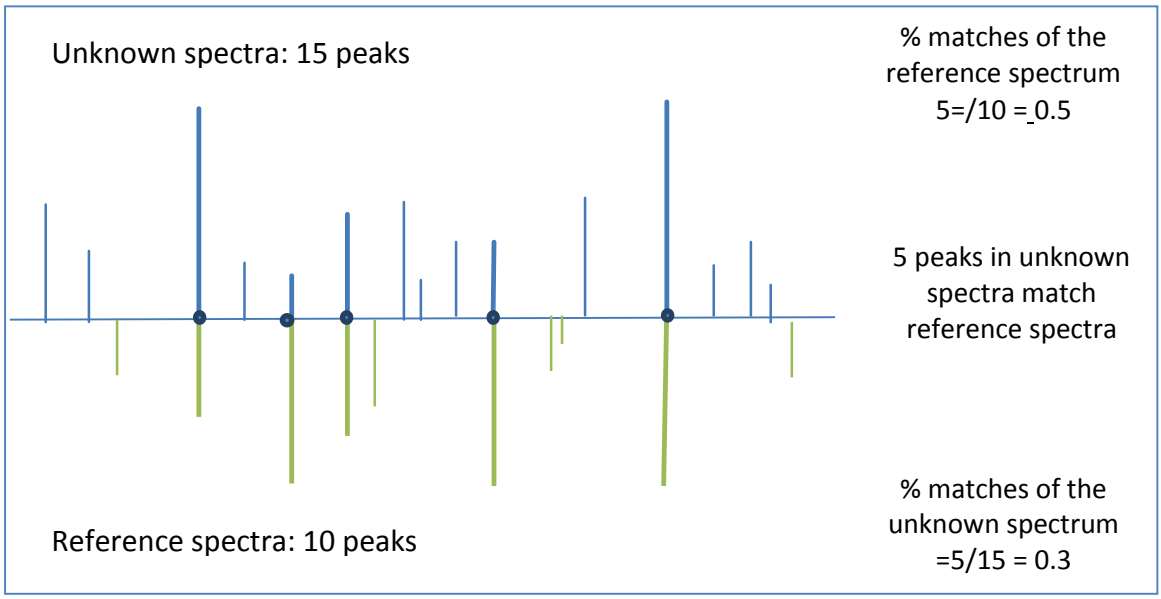


Figure 5-2: Example of the comparison of MALDI-TOF-MS analysis acquired spectrum of an unknown bacterial sample to a reference spectrum in manufacturer's bacteria database for the calculation of identification scores.

An overall score in the range from 0 (no match) to 1000 (perfect match) is derived which is transformed to a log score between 0 and 3. According to the manufacturer's guidelines, a score > 2.3 is a highly probable identification to species level, a score > 2.0 is a probable identification to species level and a score between 1.7 and 1.999 is a highly probable identification to genus level. A microorganism cannot be identified if a score < 1.7 is achieved. In this study only scores > 2.3 were accepted for identification. All samples were spotted in duplicate on MALDI targets and each MALDI target contained control strains also spotted in duplicate.

5.2.7 Antibiotic susceptibility testing method

Amoxicillin and ciprofloxacin minimum inhibitory concentration values (MICs) for 229 *E.coli* isolates and 129 *E.faecium* isolates were assessed using antibiotic gradient strips (Etest®, Biomerieux) according to manufacturer instructions. In addition, cefpodoxime MIC values for 187 *E.coli* isolates, clarithromycin MIC values for 129 *E.faecium* isolates and vancomycin MIC values for 109 *E.faecium* isolates were also assessed. There are different antibiotic susceptibility methods available (see Chapter 3, Section 3.4). However, gradient strips produce minimum inhibitory concentration values (the lowest concentrations of an antibiotic that will inhibit visible growth of a microorganism) and are easy to interpret.

Each gradient strip holds a predefined stable gradient of 15 two-fold (log_2) antibiotic concentrations. The concentration gradients for amoxicillin, cefpodoxime, clarithromycin and vancomycin spanned the range, 0.016 – 256 mg/L. The ciprofloxacin concentration gradient was between 0.002 and 32 mg/L. For each isolate under investigation, a bacterial suspension was made from colonies taken from a fresh overnight culture emulsified in 0.85 % NaCl sterile solution to achieve inocula turbidity comparable to a 0.5 McFarland standard solution (0.05 mL of 1.175% barium chloride dihydrate and 9.95 mL of 1% sulphuric acid).

The inoculums were applied to 140 mm Mueller Hinton agar petri dishes (50 mL of Mueller Hinton agar in each plate to give a depth of 4 mm) with a sterile swab, covering the entire surface to ensure a continuous bacterial growth with no discrete colonies (confluent growth). The plates were allowed to dry for approximately 15 min before the antibiotic gradient strips were aseptically applied with tweezers and the plates incubated at 37 °C for 16-20 h. After the required incubation period, the MIC values for bactericidal (amoxicillin, ciprofloxacin, cefpodoxime and vancomycin) antibiotics were read directly from the point where the edge of the inhibition ellipse intersected the side of the antibiotic gradient strips (shown by the red arrow in Figure 5-3).

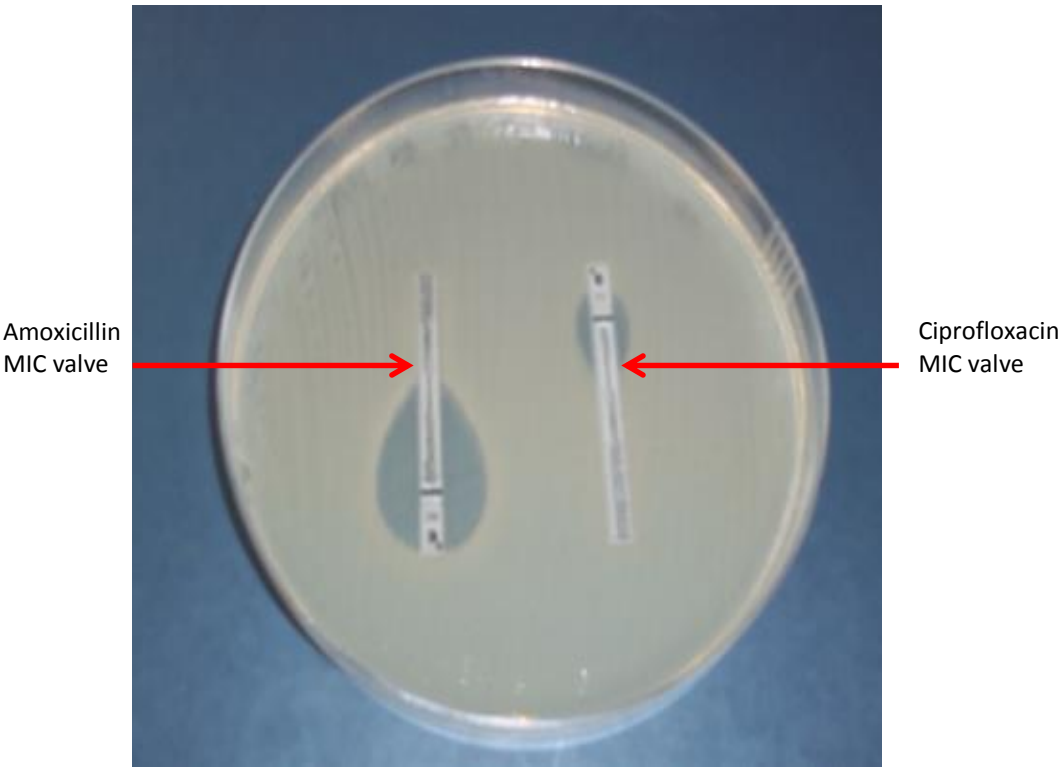


Figure 5-3: The measurement of amoxicillin and ciprofloxacin MIC values using antibiotic

gradient strips. The MIC value is observed where the edge of the ellipse of inhibition intersects the antibiotic gradient strip as shown by the red arrow.

5.2.7.1 Interpretation of minimum inhibitory concentration values (MIC)

In this study, the antibiotic concentrations used to distinguish between resistant and sensitive bacterial strains used for treatment purposes in clinical settings will be referred to as resistant and sensitive clinical breakpoint values (CBPs) respectively. The antibiotic concentrations that are used to distinguish between wild type (WT) bacteria that do not harbour acquired or mutational resistance and non-wild type (NWT) bacteria that have acquired resistance will be referred to as epidemiological cut off values (ECOffs).

The CBP and ECOff values used to interpret the MIC values obtained for *E.coli* isolates are given in Table 5-3 and were taken from EUCAST (2012).

Table 5-3: *E.coli* CBP and ECOff values for selected antibiotics

	Clinical breakpoint values (mg/L)		Epidemiological cut off values (mg/L)
	Resistant	Sensitive	Wild type
Amoxicillin	> 8	≤ 8	≤ 8
Ciprofloxacin	> 1	≤ 0.5	≤ 0.064
Cefpodoxime	> 1	≤ 1	≤ 2

Values taken from EUCAST (2012)

The CBP and ECOff values used to interpret the MIC values obtained for *E.faecium* isolates are given in Table 5-4 and were also taken from EUCAST (2012).

Table 5-4: E.faecium CBP and ECOff values for selected antibiotics

	Clinical breakpoint values (mg/L)		Epidemiological cut off values (mg/L)
	Resistant	Sensitive	Wild type
Amoxicillin	> 8 mg/L	≤ 4 mg/L	ND
Ciprofloxacin	> 4 mg/L	≤ 4 mg/L	WT ≤ 4 mg/L
Clarithromycin	ND	ND	WT ≤ 4 mg/L
Vancomycin	> 4 mg/L	≤ 4 mg/L	WT ≤ 4 mg/L

All values taken from EUCAST (2012). ND – not determined.

5.2.8 Reference control strains

The reference control strains used were obtained in ampules from the National Collection of Type Cultures (NCTC), Public Health England, UK and from the National Collection of Industrial Food and Marine Bacteria (NCIMB) and in the form of Culti-loops® (Remel, Thermo Scientific, UK). The reference control strains used are outlined in Table 5-5 and were analysed in parallel with the isolated samples. The control strains recommended by the British Antimicrobial Susceptibility Committee (BSAC, 2011) for antibiotic susceptibility testing were used. For the performance of the antibiotic tests to be acceptable the minimum inhibitory concentration values (MICs) for control strains should be within one two-fold dilution (log_2) of the expected MIC values (BSAC, 2011).

Table 5-5: Reference bacteria control strains used in the methods for detection, identification of bacteria and for antibiotic susceptibility testing.

	Reference strain	Microorganism	Test details
Growth media reference strains	NCTC 10416	*Escherichia coli*	To ensure if *E.coli*/coliform chromogenic agar facilitated the growth of *E.coli* To assess if pseudomonas agar and Slanetz and Bartley inhibited non-target bacteria (e.g. *E.coli*)
	NCTC 10662	*Pseudomonas aeruginosa*	To ensure pseudomonas agar facilitated the growth of *Pseudomonas aeruginosa* To assess if *E.coli*/coliform chromogenic agar inhibited non-target bacteria (e.g. *Pseudomonas aeruginosa*)
	NCTC 775	*Enterococcus faecalis*	To ensure Slanetz and Bartley selective growth media facilitated the growth of *E.faecalis*
	NCTC 6571	*Staphylococcus aureus*	To ensure mannitol salt agar facilitated the growth of *Staphylococcus aureus*
	NCTC 10416	*Escherichia coli*	To assess if mannitol salt agar inhibited non-target bacteria (e.g. *E.coli*)
MALDI-TOF identification reference strains	NCIMB 8023	*Leuconostoc mesenteroides*	To monitor the performance of using MALDI-TOF-MS for the identification of enterococci species (and phenotypically similar bacteria) and *E.coli*
	NCIMB 700814	*Pediococcus pentosaeceus*	
	NCTC 35667	*Enterococcus faecium*	
	NCTC 775	*Enterococcus faecalis*	
	NCTC 12361	*Enterococcus casseliflavus*	
	NCTC 12368	*Enterococcus Hirae*	
	NCTC 12359	*Enterococcus gallinarum*	
	NCTC 10416	*Escherichia coli*	
Antibiotic susceptibility testing	NCTC 10416	*Escherichia coli*	Amoxicillin, cefpodoxime and ciprofloxacin minimum inhibitory concentrations values (MICs) expected are 2.0, 0.25 and 0.015 mg/L respectively
	NCTC 6571	*Staphylococcus aureus*	Amoxicillin, ciprofloxacin and clarithromycin MICs expected are all 0.12 mg/L. The MIC expected for vancomycin is 0.5 mg/L (sensitive)

5.2.9 Repeated sub-culture of resistant isolates

E.coli and *E.faecium* isolated from settled sewage, final treated effluent and surface waters both up- and down-stream of the final effluent discharge point identified as resistant (using methods given in section 5.2.7) were sub-cultured repeatedly and retested for antibiotic susceptibility to the same antibiotics. A selection of fresh overnight cultures from preserved stocks of resistant isolates (nutrient broth with 20 % glycerol and stored at -80 ° C) were sub-cultured onto nutrient agar on ten separate daily occasions (incubated overnight at 30 °C). From the tenth sub-culture, colonies were selected for antibiotic susceptibility testing using the methods given in section 5.2.7. Isolates were considered to have maintained resistance if the MIC values determined following repeated sub-culture were within one two-fold dilution of the originally defined MIC.

5.2.10 One and two proportions statistical analysis

Using the clinical breakpoints and epidemiological cut off values, the proportions (%) of *E.coli* and *E.faecium* isolates at each sampling point (settled sewage, final treated effluent, up- and down-stream surface water from the treated effluent discharge point) resistant to selected antibiotics were estimated. The 1-proportion test was used to calculate the 95.0 % confidence interval for the estimated proportions of resistance. The 2-proportions test (Fishers exact test) was used to compare the difference of the proportions of resistance between sampling points. All statistical analysis was performed using Minitab software v. 16.

5.3 Wastewater and surface water bacterial analysis

5.3.1 Enumeration of indicator bacteria in wastewater and surface waters

Microbiological analysis of the settled sewage samples indicated that the levels of heterotrophic bacteria were in the range 8.8 – 9.7 $\log_{10}$ CFU/100 mL compared to total coliforms, *E.coli* and enterococci which were present in the ranges 7.0 -7.2 $\log_{10}$ CFU/100 mL, 6.7 -7.0 $\log_{10}$ CFU/100 mL and 5.9 – 6.7 $\log_{10}$ CFU/100 mL, respectively (Table 5-6). Typically *E.coli* are present in municipal sewage at concentrations 10 to 100 times higher than enterococci (Sinton et al., 1993) as human faeces contain greater concentrations of *E.coli* (up to 10^9 per gram of faeces) compared to enterococci (up to 10^6 per gram of faeces) (Environment Agency, 2002) and therefore the higher *E.coli* concentrations present in the settled sewage were expected.

Staphylococci and pseudomonas were found to be in lower concentrations at approximately 4 - 5 $\log_{10}$ CFU/100 mL. Heterotrophic bacteria, total coliforms, *E.coli*, enterococci, staphylococci and pseudomonads were present at lower concentrations in the final treated effluent. Activated sludge treatment had reduced the enumerated organisms by > 97 % corresponding to decreases in the enumerated organism concentrations in the range 1.8 – 3.2 $\log_{10}$ CFU/mL (Table 5-7).

Table 5-6: [a] Enumeration of bacteria in wastewater effluents and surface waters.

	Settled sewage	Final effluent	Up-stream	Down-stream
	Mean ± standard deviation Log_{10} CFU/100 mL			
Heterotrophic	9.4 ± 0.4	7.5± 0.7	6.5 ± 0.6	7.4 ± 0.20
bacteria	(8.8 – 9.7)	(6.8 – 8.1)	(6.2 – 6.8)	(7.1 – 7.3)
Total coliforms	7.1 ± 0.1	4.8 ± 0.7	3.8 ± 0.1	5.0 ± 0.5
	(7.0 – 7.2)	(4.6 – 4.9)	(3.6 – 4.00)	(4.3 -5.2)
E.coli	6.8 ± 0.2	4.4 ± 0.4	2.9 ± 0.2	4.4 ± 0.6
	(6.7 – 7.0)	(4.4 – 4.9)	(2.7 – 3.1)	(3.6 – 5.1)
enterococci	6.2 ± 0.2	3.0 ± 0.2	1.9 ± 0.1	2.9 ± 0.2
	(5.9 – 6.7)	(2.9 – 3.1)	(1.8 – 2.0)	(2.5 – 3.2)
staphylococci	4.7± 0.2	2.6 ± 0.1	1.7 ± 0.3	2.5 ± 0.5
	(4.5 – 4.9)	(2.5 – 2.6)	(1.3 – 1.7)	(1.4 -2.9)
pseudomonas	4.8± 0.3	2.5 ± 0.3	1.4 ± 0.2	2.1 ± 0.6
	(3.9 – 5.0)	(1.8 – 2.7)	(< lod -1.6)	(1.0 -2.3)

[a] enumerations corrected using confirmation test results. Mean value of five sampling occasions. The concentration range is given in the parenthesis.

Typically, of the order of one log_{10} unit higher concentrations of total heterotrophic bacteria, total coliforms, *E.coli*, enterococci, staphylococci and pseudomonas were detected in the surface waters down-stream of the wastewater treatment plant discharge point compared to the up-stream point. The concentration of the enumerated bacteria in the final treated effluent was similar (2-Sampled T test; $P > 0.05$) to those detected in the surface waters down-stream of the treated effluent discharge point.

Table 5-7: Reduction of indicator bacteria during activated sludge treatment

	Mean % reduction ± standard deviation	Mean Log_{10} reduction ± standard deviation
Total heterotrophic bacteria	97.8 ± 1.5	1.8 ± 0.4
Total coliforms	99.4 ± 0.2	2.3 ± 0.2
E.coli	99.5 ± 0.2	2.4 ± 0.3
enterococci	99.9 ± 0.1	3.2 ± 0.3
staphylococcus	99.2 ± 0.4	2.1 ± 0.2
Pseudomonas	99.4 ± 0.2	2.2 ± 0.2

Mean reduction calculated from the mean bacterial concentrations observed in settled sewage and final treated effluent from five sampling occasions. % reduction = [settled sewage] – [final treated effluent] / [settled sewage] x 100.

5.3.2 Evaluation of detection and enumeration growth media to detect indicator bacteria in wastewater and surface water

5.3.2.1 Escherichia coli and total coliforms

The importance of coliform bacteria and *E.coli* as indicators of water quality is described in Chapter 3, Section 3.1.1. To assess the effectiveness of using chromogenic agar, based on β-galactosidase and β- glucuronidase enzymatic activity, for the selective and differential detection of coliforms and *E.coli* in wastewater and surface water, fifty purple colonies (presumptive *E.coli*) and thirty pink colonies (presumptive coliforms other than *E.coli*) were isolated and identified using a commercial biochemical identification kit (API 20E) in addition to the biochemical confirmation tests (indole and oxidase). The results are given in Table 5-8. MALDI-TOF-MS analysis was not available for this part of the study.

Table 5-8: [a] Phenotypic identification (Biochemical kits) to species level of presumptive *E.coli* and coliform bacteria isolated from wastewaters and surface waters grown on *E.coli*/coliform chromogenic agar.

Species	No. of presumptive *E.coli* (% of total presumptive E.coli)		No. of presumptive coliforms (% of total presumptive coliforms)	
	IND^+	IND^-	OX^-	OX^+
Escherichia coli	44 (88.0)		[d] 2 (6.7)	
Serratia odorifera			3 (10.0)	
Enterobacter cloacae			5 (16.7)	
Klebsiella pneumoniae			3 (10.0)	
Klebsiella oxytoca			2 (6.7)	
Citrobacter freundii		1 (2.0)	2 (6.7)	
Raoultella orninthinolytica	1 (2.0)			
Pantoea spp	1 (2.0)			
Aeromonas spp				7 (23.3)
Unidentified	2 (4.0)	1 (2.0)	2 (6.7)	4 (13.3)
Total	48 (96.0)	2 (4.0)	19 (63.3)	11 (36.7)
Total	[b] 50		[c] 30	

[a] MALDI-TOF-MS was not available at this point of the study to identify presumptive bacteria, [b] total presumptive *E.coli,* [c] total presumptive coliforms, [d] Indole positive

Forty eight presumptive *E.coli* isolates (96.0% of total) produced indole from tryptophan at 44 °C (IND^+) which is characteristic of *E.coli*. Three presumptive *E.coli* isolates could not be identified to species or even genus level using the commercial biochemical kit. Two (4.0%) of the unidentified isolates produced indole from tryptophan at 44 °C (IND^+) and one isolate

(2.0%) did not (IND^-). Three presumptive *E.coli* isolates were identified as Enterobacteria (including *Citrobacter freundii*) other than *E.coli*. *E.coli* false positive results caused by *Citrobacter* species were also found in a similar study by Alonso et al. (1996). From a total of forty eight presumptive *E.coli* colonies that produced indole from tryptophan at 44 °C (IND^+), forty four isolates were successfully identified as *E.coli*. Therefore an *E.coli* confirmation rate of 91.6% was achieved when using this chromogenic agar in combination with testing for indole production at 44 °C. Thus, the use of the chromogenic agar in combination with indole confirmation was considered suitable to detect and enumerate *E.coli* from environmental waters.

From the thirty presumptive coliform isolates tested, nineteen were oxidase negative (do not produce cytochrome oxidases) and eleven pink isolates produced a positive oxidase reaction (OX^+) not characteristic of the coliform group. Seventeen oxidase negative (OX^-) isolates were identified as *Serratia odorifera* (10.0%), *Enterobacter cloacae* (16.7%), *Citrobacter freundii* (6.7%), *Klebsiella oxytoca* (6.7%) and *Klebsiella pneumoniae* (10.0%) and two isolates were identified as *Escherichia coli* giving a *E.coli* false negative rate of 6.7%. Olson et al. (1991) reported that *E.coli* false negatives can occur due to cell injury, cell impermeability or inability of the cell to metabolise the chromogenic substrate and lack of gene expression. However, these two *E.coli* isolates did produce indole at 44 °C (IND^+). Therefore, from a total of seventeen OX^-, IND^- negative isolates, fifteen could be successfully identified as bacteria belonging to the coliform group giving a coliform confirmation rate of 88.2%.

To be able to study the impact of wastewater on receiving surface waters, bacteria of faecal origin were desirable. *E.coli* bacteria are predominantly of faecal origin (Environment Agency, 2000) and were easily detected (91.6 % confirmed) using this chromogenic agar. In addition, the concentrations of *E.coli* detected in the settled sewage, treated effluent and surface waters (See Section Table 5-6) were high enough to investigate the proportions of antibiotic resistance within this species. Therefore, *E.coli* bacteria were selected for subsequent antibiotic susceptibility testing. Conversely, coliform bacteria were not selected for subsequent antibiotic resistance testing despite the high concentrations detected in the water samples. This is because of the diversity of bacterial species within the coliform group taken from the water samples in which some species are not necessarily of faecal origin (e.g. *Citrobacter freundii*). In addition, antibiotic resistance may be more predominant in some species of the coliform group than others. Therefore coliforms as a group were not selected to investigate the transfer of resistance from wastewaters to surface waters.

5.3.2.2 Staphylococcus

To evaluate the use of mannitol salt agar to detect *Staphylococcus* including *Staphylococcus aureus* isolated from environmental waters, eighty eight colonies were sub-cultured for confirmation tests (Gram staining and catalase) and identified using a commercial biochemical kit (API Staph®). It was difficult to differentiate between presumptive cream coloured *Staphylococcus aureus* colonies and staphylococci pink coloured colonies particularly with higher bacterial counts. This was due to the fermentation of the mannitol masking the colour of the pink colonies and therefore this media could not be used for the

differentiation of *Staphylococcus aureus* from other staphylococci species in environmental waters.

All eighty eight colonies confirmed as Gram positive, however 26 presumptive staphylococci isolates confirmed as catalase negative and therefore are not characteristic of the staphylococci. These isolates were not tested any further. A total of 16 (25.8 %) of the confirmed (Gram stain and catalase) staphylococci isolates could not be identified using the commercial biochemical kit and 22 isolates (35.5 %) could only be identified to genus levels (all staphylococci), leaving 24 isolates (38.7 %) that could be identified to species level (Table 5-9).

The different staphylococci species identified are given in Table 5-9. The diversity of the species was expected as they are ubiquitous bacteria reported as part of the normal microbiota associated with air, soil, water, humans and other animals (Faria et al., 2009; Zadoks et al., 2009; Resch et al., 2011). However, due to the difficulty in differentiating *Staphylococcus aureus* using mannitol salt agar membrane filtration and the difficulty in identifying staphylococci species from environmental waters using API Staph®, monitoring the antibiotic resistance profiles of staphylococci species was not carried out.

Table 5-9: Identification of presumptive staphylococci species isolated from environmental waters using API Staph®.

Identification	Gram and catalase positive cream colonies	Gram positive and catalase negative

Staphylococcus cohnii	2 (3.2 %)	
Staphylococcus sciuri	16 (25.8 %)	
Staphylococcus saprophyticus	2 (3.2 %)	
Staphylococcus aureus	2 (3.2 %)	
Staphylococcus xylosus	2 (3.2 %)	
Identified to genus only	22 (35.5 %)	
Unidentified	16 (25.8 %)	26
Total	[a]62	[b]26

The % of total Gram and catalase positive presumptive *Staphylococcus aureus* colonies are given in the parenthesis. [a] of the total 88 colonies, 62 confirmed as staphylococci. [b] 28 colonies not characteristic of staphylococci genera.

5.3.2.3 Pseudomonas

In total eighty six presumptive pseudomonas isolates (twenty nine red/brown, thirty four blue/green and twenty three cream coloured colonies) were sub-cultured for further confirmation (oxidase) and phenotypic identification tests (API NE®). From surface waters up-stream of the treated effluent discharge point, typically only cream coloured colonies grew on the membrane filters regardless of the range of sample dilutions. Five cream coloured colonies (5.8 % of total isolated) were not oxidase positive and therefore were not further identified. It has been reported that pseudomonas agar base can support the growth of *Acinetobacter* species which are typically found in the environment and are oxidase negative (Casanovas-Massana, 2006). All red/brown and blue/green colonies were oxidase positive and therefore tested using the commercial biochemical kit (Table 5-10).

Table 5-10: Identification of presumptive pseudomonas isolated from environmental waters using API 20 NE®

	Red/brown colonies	Cream coloured colonies	Blue/green colonies
Pseudomonas aeruginosa			15 (17.4)
[a] pseudomonas	11 (12.8)	13 (15.1)	9 (10.4)
Unidentified	18 (20.9)	5 (5.8)	10 (11.6)
Oxidase –ve (not identified)		5 (5.8)	
Total		86	

[a] identified to genus level only. All oxidase positive unless stated. -ve = negative. The % of total isolates is given in the parenthesis.

Only 15 (17.4 %) of the isolates (all from blue/green coloured colonies) could be identified to species level and all were identified as *Pseudomonas aeruginosa*. Thirty three isolates could be identified to genus levels, all belonging to the pseudomonas genera. However, thirty three isolates could not be identified using the commercial biochemical identification system. The difficulty in identifying the presumptive pseudomonas isolates to species level, emphasises that phenotypic identification is challenging and thus in this study it also makes it difficult to assess the use of pseudomonas agar (with supplement) for the selective isolation of *Pseudomonas aeruginosa* from environmental waters. Therefore antibiotic resistance profiles of *Pseudomonas aeruginosa* in environmental waters were not monitored further in this work.

5.3.2.4 Enterococci

Enterococci include a number of different species that occur in the faeces of humans and warm-blooded animals and can therefore provide an indication of such pollution. In addition, high numbers of enterococci were detected in the collected water samples (see Section 5.3.1) and therefore enterococci were considered as ideal representative organisms to study the transfer of antibiotic resistant bacteria from wastewater to surface waters. Although enterococci are easily isolated on Slanetz and Bartley media, the distinction between the different species is difficult as they produce colonies of similar appearance. Therefore additional tests were required to identify presumptive enterococci to species level. There are different methods available to identify bacteria to species level including biochemical methods and mass spectrometry (Chapter 3, section 3.2.3). In this study the API 20 Strep biochemical profile system was initially used to identify presumptive enterococci *isolates* (n = 61) from waste and surface waters to species levels (Table 5-11). The same isolates were then identified using matrix assisted laser desorption ionisation time of flight mass spectrometry analysis (MALDI-TOF-MS) when the instrument became available.

Only 30 presumptive enterococci isolates (49.2 %) could be identified to species level using the commercial kit, whilst the identification to species level of all 61 isolates was achieved using MALDI-TOF-MS analysis. When comparing the results from phenotypic identification to MALDI-TOF-MS identification, five discordant identifications occurred. However, atypical enterococci strains can be misidentified as *Lactococcus* species using biochemical systems (Facklam et al., 1989) and species such as *E.hirae* are difficult to identify phenotypically due

to the variation of sugar fermentation profiles observed within the species (Arias et al., 2006).

Table 5-11: Identification of presumptive enterococci isolates using API 20 Strep biochemical system and MALDI-TOF-MS analysis

Enterococci spp	[a] No. of presumptive enterococci isolates			
	Identified with MALDI-TOF	Identification with API 20 Strep	Discordant identification	Not identified with API 20 Strep
Enterococcus faecium	40 (65.6 %)	22	[b]5	13
Enterococcus faecalis	4	4	0	0
Enterococcus duran	3	1	0	2
Enterococcus hirae	5	0	[c]2	3
Enterococcus mundtii	1	0	0	1
Aerococcus virridan	3	3	0	0
Escherichia coli	5	0	0	5 (8.2 %)
Total	61	30 (49.2)	7 (11.5)	24 (39.3)

[a] Maroon colonies isolated from Slanetz and Bartley media using the membrane filtration method given in Section 5.2.4. Using the biochemical system, isolates identified but not consistent with MALDI-TOF identification were [b] *Lactococcus lactis* and [c] *Enterococcus faecium*. The % of total isolates is given in the parentheses.

The biochemical tests included in the phenotypic system do not include tests to identify Gram negative bacteria and this therefore explains why five isolates (8.2 %) could not be identified using this test kit. It was decided that identification of presumptive enterococci isolates using the commercial systems was not sufficiently reliable for this study.

Conversely, for routine isolates, MALDI-TOF-MS analysis has shown overall correct identification levels often greater than 99 % (Bizzini et al., 2011; Eigner et al., 2009; Cherkaoui et al., 2010) when compared to 16S rRNA Gene Sequencing for Identification (considered as the 'gold standard' method for identification) and therefore selected for this work.

A total of 262 presumptive enterococci isolates taken from wastewater and surface water were sub-cultured onto *E.faecium* chromogenic agar to assess if the media could differentiate *E.faecium* from the other species of enterococci. 112 of presumptive *E.faecium* isolates producing green colonies were identified as *E.faecium* (73.2 %). However, 41 (26.8 %) of the isolates producing green colonies were identified as enterococci species other than *E.faecium* giving a false positive rate of 26.8 % (Table 5-12).

30 of the isolates producing blue colonies (presumptive other enterococci species) were identified as *E.faecium* giving a false negative rate of 27.5 %. Ford et al. (1994) reported that *E. mundtii*, *E.gallinarum* and *E.casseliflavus* can ferment arabinose and therefore can be misidentified as *E.faecium* using cephalextin arabinose agar. Due to the high false positive and negative rate using this media that may lead to misidentification of presumptive enterococci species to species level, this chromogenic agar was not used as a confirmation test for subsequent work in this study. Therefore, for subsequent work in this chapter, the identification of presumptive enterococci (grown on Slanetz and Bartley media) to species level was performed using MALDI-TOF-MS analysis.

Table 5-12: Assessment of a chromogenic media to differentiate *Enterococcus faecium* from other enterococci species.

[d] Identification	[a] No. of isolates	
	Green colonies (presumptive *E.faecium*)	Blue colonies (presumptive enterococci)
Enterococcus faecium	112 (73.2)	30 (27.5)
Enterococcus faecalis	11 (7.2)	36 (33.0)
Enterococcus casseliflavus	2 (1.3)	1 (0.9)
Enterococcus gallinarum	2 (1.3)	0
Enterococcus hirae	4 (2.6)	29 (26.6)
Enterococcus mundtii	1 (0.7)	1 (0.9)
Enterococcus durans	1 (0.7)	1 (0.9)
Enterococcus avium	0	1 (0.9)
Escherichia coli	20 (13.1)	5 (4.6)
Aerococcus virrdan	0	3 (2.8)
Streptococcus equinus	0	1 (0.9)
Total	[b]153	[c]109
Total false positives	41 (26.8)	
Total false negatives		30 (27.5)

[a] originally detected on Slanetz and Bartley media using membrane filtration. [b] Total number of green colonies. [c] Total number of blue colonies. [d] identification carried out with MALDI-TOF-MS. The % of total isolates given in parentheses.

5.3.2.5 Distribution of enterococci species in wastewater and surface waters

The distribution of the enterococci species identified in the environmental waters sampled for this study are shown in Figure 5-4. By far the most abundant species identified in the settled sewage, final treated effluent and surface water down-stream of the treated effluent discharge point was *E.faecium*, followed by *E.faecalis* and then *E.hirae*. Therefore *E.faecium*

was selected as a representative species to study the transfer of antibiotic resistance from wastewater to surface water.

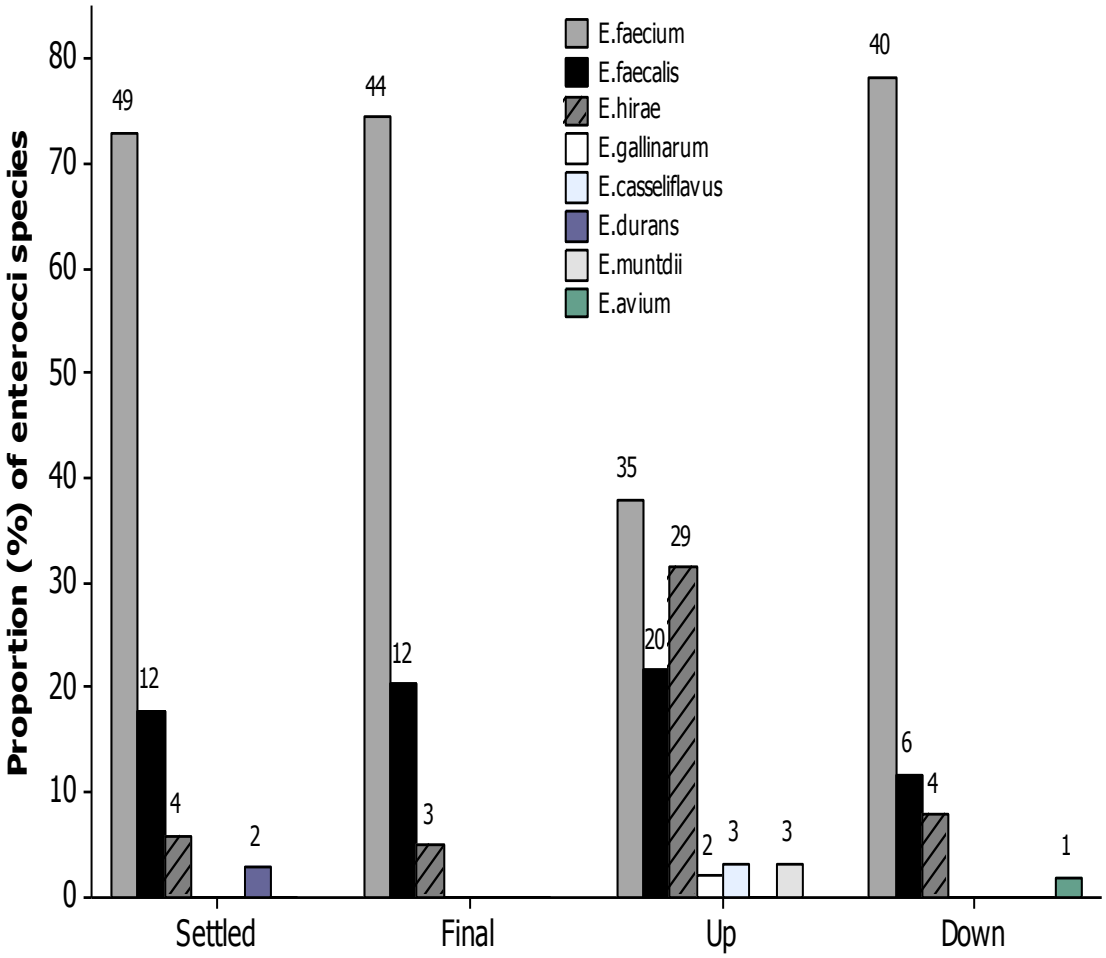


Figure 5-4: The distribution (%) of enterococci species in settled sewage (Settled), final treated effluent (Final), surface water up-stream (Up) and down-stream (Down) of the wastewater treatment plant discharge point. The number of isolates in each species group is given above each bar.

The other species detected included, *E. durans*, *E. avium*, *E. muntdii*, *E.gallinarum* and *E.casseliflavus*. Significantly lower proportions (2-proportions test; $P < 0.001$) of *E.faecium* were identified in the surface water up-stream of the treated effluent discharge point

compared to the other sampling points (Table 5-13). Elevated levels (2-proportions test; P < 0.001) of *E.hirae* were detected in the surface water up-stream of the final effluent discharge point compared to the levels at other sampling points.

Table 5-13: The proportion (%) of the most prevalent of enterococci species identified in the environmental waters sampled for this study.

	E.faecium (%)	*E.faecalis* (%)	*E.hirae* (%)
Settled sewage	73.1 (60.9 – 83.2)	17.9 (9.6 – 29.2)	6.0 (1.7 – 14.6)
Final effluent	74.6 (61.6 – 85.0)	20.3 (11.0 – 32.8)	5.1 (1.1 – 14.1)
Down-stream	78.6 (64.7 – 88.7)	11.8 (4.4 – 23.9)	7.8 (2.2 -18.9)
Up-stream	[a]38.5 (28.4 – 49.2)	22.0 (14.0 – 31.9)	[b]31.8 (22.5 - 42.5)

1-proportion 95 % confidence intervals are given in the parenthesis. [a] 2-proportions test of the proportion of *E.faecium* between samples; p < 0.001. [b] 2-proportions test between *E.hirae* proportions; P < 0.001).

5.3.3 Antibiotic resistance in *Escherichia coli* from environmental waters

5.3.3.1 Escherichia coli MIC determination

Amoxicillin and ciprofloxacin minimum inhibitory concentrations for 229 *E.coli* isolates recovered from the settled sewage (n = 60), final treated effluent (n = 56) and surface waters both up-(n = 59) and down-stream (n = 54) relative to the treated effluent discharge point were measured and are presented in Table 5-14. In addition, cefpodoxime minimum inhibitory concentrations were measured for a total of 187 *E.coli* isolates, which were similarly distributed between the four sampling sites, and are also presented in Table 5-14.

Table 5-14: Amoxicillin, cefpodoxime and ciprofloxacin minimum inhibitory concentration values (mg/L) for *E.coli* isolated from wastewaters and surface waters

Antibiotic	Sample point	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Amoxicillin	Settled sewage (n = 60)	2-256	4	256
	Final effluent (n = 56)	2-256	4	256
	Up-stream (n = 59)	1-256	4	8
	Down-stream (n = 54)	1-256	4	256
Ciprofloxacin	Settled sewage (n = 60)	0.002-32	0.012	0.190
	Final effluent (n = 56)	0.002-32	0.008	0.125
	Up-stream (n = 59)	0.002- 0.03	0.008	0.016
	Down-stream (n = 54)	0.002-4	0.008	0.064
Cefpodoxime	Settled sewage (n = 60)	0.19–24	0.5	0.75
	Final effluent (n = 56)	0.19- 256	0.5	1
	Up-stream (n = 59)	0.006-1	0.5	1
	Down-stream (n = 54)	0.125-2	0.38	1

MIC_{50} is equivalent to the concentration of antibiotic that inhibits 50% of *E.coli*; MIC_{90} is equivalent to the concentration of antibiotic that inhibits 90% of *E.coli*.

The MIC values measured span the concentrations that define sensitive (according to clinical breakpoints) and wild type strains (according to epidemiological cut off values) up to concentrations greater than those used to define resistant and non-wild type strains. The concentrations of amoxicillin (4 mg/L), ciprofloxacin (0.008 – 0.012 mg/L) and cefpodoxime (0.38 – 0.50 mg/L) that inhibited 50 % of the *E.coli* isolates (MIC_{50}) were similar and within one two-fold dilution for all sampling points.

The concentrations of cefpodoxime that inhibited 90 % of the *E.coli* isolates in the settled sewage, final treated effluent and surface waters both up- and down-stream from the effluent discharge point were similar (0.75 – 1 mg/L) and within one two-fold dilution for all sampling points. However, the concentrations of amoxicillin and ciprofloxacin that inhibited 90 % of *E.coli* (MIC_{90}) were lower for isolates taken from the surface water up-stream of the treated effluent discharge point compared to down-stream. This is possibly indicating an

impact of the discharged wastewater treated effluent on the prevalence of amoxicillin and ciprofloxacin resistant *E.coli* within surface waters down-stream from the discharge point. No significant differences in amoxicillin, ciprofloxacin or cefpodoxime MIC values amongst sampling points were found by applying a Mann-Whitney analysis ($P > 0.05$).

5.3.3.2 Distribution of antibiotic minimum inhibitory concentration values determined for E.coli taken from wastewater and receiving surface water

The distributions of the amoxicillin MIC values measured for *E.coli* isolates taken from settled sewage, final treated effluent and surface waters both up- and down-stream of the final effluent discharge point are presented in Figure 5-5 and show that amoxicillin resistant *E.coli*, according to both the resistant CBP value ($R > 8$ mg/L) and ECOff value ($WT \leq 8$ mg/L) (Table 5-3) were present at each sampling point.

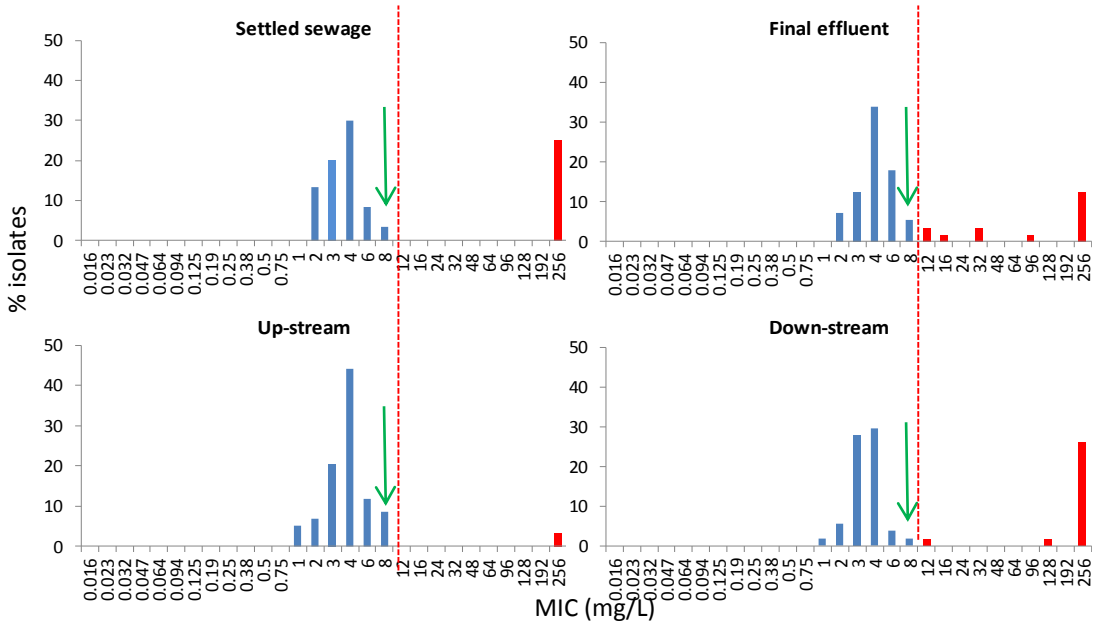


Figure 5-5: Distributions of the amoxicillin MIC values measured for *E.coli* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The red dashed line represents the clinical resistance breakpoint value (R > 8 mg/L) and the green arrow represents the epidemiological cut-off value (WT ≤ 8 mg/L). The blue bars represent wild type *E.coli* (wild type) and the red bars represent non-wild type *E.coli* (acquired resistance).

The distributions of ciprofloxacin MIC values measured for *E.coli* isolated from all sampling points are presented in Figure 5-6. *E.coli* with acquired ciprofloxacin resistance (wild type $\leq$ 0.064 mg/L) and considered resistant according to CBP values (R > 1 mg/L) were identified in the settled sewage, final treated effluent and surface water down-stream from the final effluent discharge point. No ciprofloxacin resistant or non-wild type *E.coli* isolates were detected in surface waters up-stream of the final treated effluent discharge point.

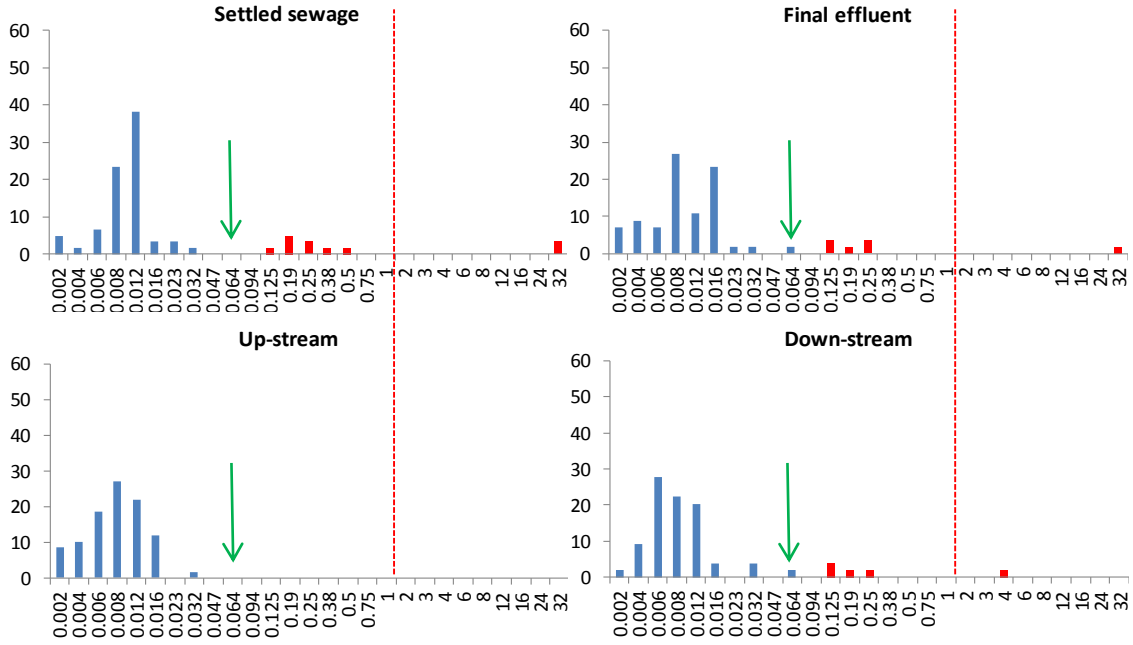


Figure 5-6: Distributions of ciprofloxacin MIC values measured for *E.coli* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The red dashed line represents the clinical resistance breakpoint value (R > 1 mg/L) and the green arrow represents the epidemiological cut-off value (WT ≤ 0.064 mg/L). The blue bars represent wild type *E.coli* (wild type) and the red bars represent non-wild type *E.coli* (acquired resistance).

The distributions cefpodoxime MIC values measured for the *E.coli* strains isolated from all sampling points are shown in Figure 5-7. Cefpodoxime resistant strains (according to the CBP value; R > 1 mg/L) were present in settled sewage, final treated effluent and surface waters down-stream of the urban wastewater treatment plant discharge point. *E.coli* isolates with cefpodoxime acquired resistance (WT $\leq$ 2 mg/L) were only detected in the settled sewage and final treated effluent. Only cefpodoxime sensitive *E.coli* strains without acquired or mutational resistance were isolated from the surface water up-stream of the treated effluent discharge point.

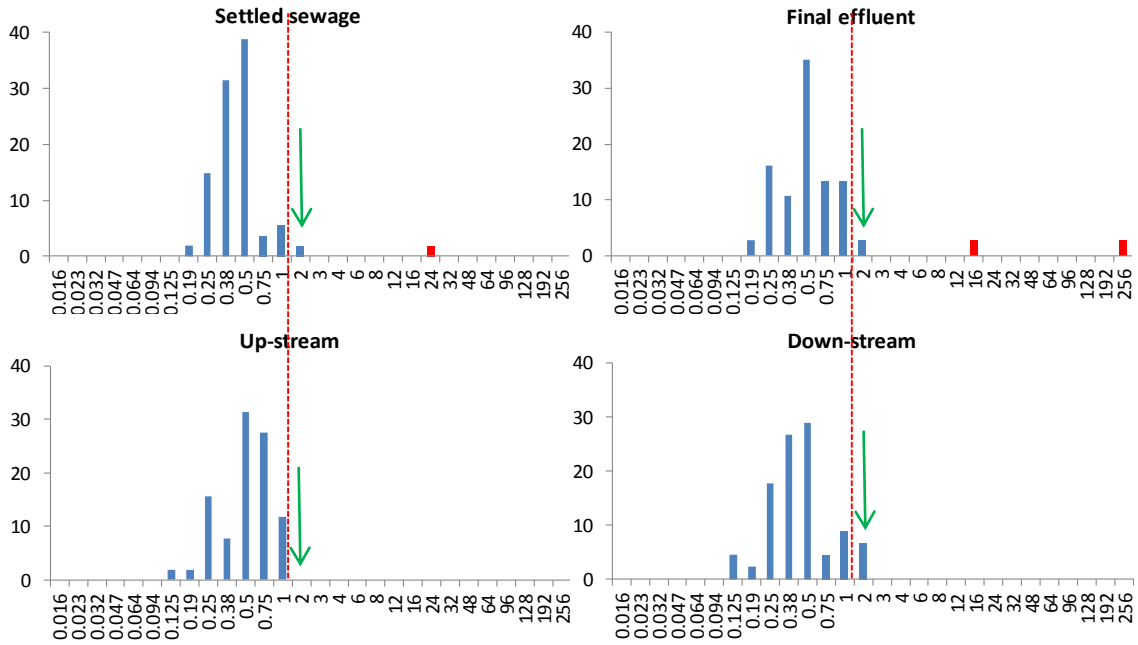


Figure 5-7: Distributions of cefpodoxime MIC values measured for *E.coli* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The red dashed line represents the clinical resistance breakpoint value (R > 1 mg/L) and the green arrow represents the epidemiological cut-off value (WT ≤ 2 mg/L). The blue bars represent wild type *E.coli* (wild type) and the red bars represent non-wild type *E.coli* (acquired resistance).

5.3.3.3 The proportion of E.coli resistant to antibiotics in wastewaters and receiving surface water

Overall, a high prevalence of *E.coli* taken from waste and surface waters, which were resistant to amoxicillin (20.1 %), was found (Table 5-15). Only 1.8 % of the all the *E.coli* isolated were identified as ciprofloxacin resistant (according to CBP values). However, when interpreting the ciprofloxacin minimum inhibitory concentrations according to the ECOff value, 9.2 % of the total *E.coli* isolates were identified as non-wild type strains with acquired ciprofloxacin resistance. Conversely, 4.3 % of *E.coli* taken from all samples was found to be

resistant to cefpodoxime (according to CBP values) however, only 1.6 % of *E.coli* was identified as non-wild type according to the cefpodoxime ECOffs value (Table 5-15).

Table 5-15: The proportion (%) of *E.coli* isolates from four different water samples which were resistant (according to ECOffs and CBP values) to amoxicillin, ciprofloxacin and cefpodoxime.

	% *E.coli*				
	[d] Settled sewage	[d] Final treated effluent	[d] Down-stream	[d] Up-stream	[b] % of total isolates
[a] Amoxicillin ECOff	25.0 (14.7 – 37.9)	23.2 (13.0 – 36.4)	29.6 (18.0 – 43.6)	3.4 (0.4 -11.7)	20.1 (15.1 - 25.9)
CBP	25.0 (14.7 – 37.9)	23.2 (13.0 – 36.4)	29.6 (18.0 – 43.6)	3.4 (0.4 – 11.7)	20.1 (15.1 - 25.9)
Ciprofloxacin ECOff	16.7 (8.3 – 28.5)	10.7 (4.0 – 21.9)	9.3 (3.1 – 20.3)	ND	9.2 (5.8 -13.7)
CBP	3.3 (0.4 – 11.5)	1.8 (0.04 – 9.5)	1.9 (0.1 – 9.9)	ND	1.8 (0.5 – 4.4)
Cefpodoxime ECOff	1.9 (0.1 – 10.0)	5.4 (0.6 – 18.2)	ND	ND	1.6 (0.3 – 4.6)
CBP	3.7 (0.5 – 12.8)	8.1 (1.7 – 21.9)	6.7 (1.4 – 18.3)	ND	4.3 (1.9 – 8.3)
[c] Multi antibiotics ECOff	10.0 (3.8 – 20.5)	5.4 (1.1 – 14.9)	5.6 (1.2 – 15.4)	None	5.2 (2.7 – 9.0)
CBP	3.3 (0.4 – 11.5)	3.6 (0.4 – 12.3)	5.6 (1.2 – 15.4)	None	3.1 (1.2 – 6.2)

1 - proportion 95 % confidence intervals (Fisher's exact test) are given in the parentheses. ND = none detected. [a] significantly different proportions (2-proportions test; $P < 0.05$) amongst sampling points. [b] % of total isolates from all sample points (i.e settled sewage, treated effluent, and surface water up- and down-stream of treated effluent discharge point). In total from all sample points, 229 isolates were tested for amoxicillin and ciprofloxacin susceptibility. A total of 187 isolates from settled sewage, treated effluent and surface water were tested for susceptibility to cefpodoxime. [c] Multi antibiotics = % isolates with acquired resistance (according to ECOff values) or resistant (according to CBP values) to more than one of the antibiotics tested. [d] The number of isolates tested for amoxicillin and ciprofloxacin susceptibility in settled sewage, treated effluent and surface water both up- and down-stream was 60, 56, 54 and 59 respectively. However for cefpodoxime the number of isolates tested were 54, 37, 51 and 45 respectively.

Overall, the levels of resistance to amoxicillin, ciprofloxacin and clarithromycin (according to ECOff values) were similar (2-proportions test; P > 0.05) in the settled sewage, final treated effluent and surface water down-stream from the effluent discharge point. However, in the surface water up-stream from the effluent discharge point, lower levels of resistant *E.coli* were found (Table 5-15). When interpreting the measured cefpodoxime MIC values for *E.coli* according to clinical breakpoint values the levels of resistance were similar in the settled sewage, final treated effluent and surface water down-stream from the effluent discharge point. However, when using the cefpodoxime ECOff value, *E.coli* with acquired resistance to cefpodoxime were only detected in the settled sewage and final treated effluent. Only cefpodoxime wild type and sensitive *E.coli* strains were detected in the surface water up-stream from the effluent discharge point.

The proportion of *E.coli* isolates with acquired resistance (using ECOff values) or resistance (using CBP values) to more than one of the antibiotics tested were similar (2-proportions test; P > 0.05) in the settled sewage, final treated effluent and surface water down-stream from the effluent discharge point. However, lower proportions were observed when using the clinical breakpoint values compared to the epidemiological cut off values. No *E.coli* isolates with multi resistance (using ECOffs or CBP values) were identified in the surface water up-stream from the WWTP effluent discharge point.

5.3.3.4 Maintenance of resistance in E.coli

The proportion of *E.coli* isolates resistant to amoxicillin, ciprofloxacin and cefpodoxime (according to ECOff and CBP values) that maintained their resistance following ten repeated sub-cultures on antibiotic free nutrient agar are presented in Table 5-16.

Table 5-16: Proportion of resistant *E.coli* isolates (according to ECOff values and CBP values) maintaining resistance, following repeated sub-culture.

	% maintained resistance			
Resistance to	Settled sewage	Final treated effluent	Down-stream	Up-stream
[a] Amoxicillin	93.3 (15)	85.7 (14)	83.3 (12)	100.0 (2)
Ciprofloxacin (using ECOffs)	83.3 (6)	83.3 (6)	57.1 (7)	ND
(using CBPs)	100.0 (2)	100.0 (1)	100.0 (1)	ND
Cefpodoxime (using ECOffs)	100.0 (1)	100.0 (1)	ND	ND
(using CBPs)	100.0 (2)	100.0 (3)	100.0 (3)	ND

[a] ECOff and resistant CBP values are identical for amoxicillin (8 mg/L). Total number of isolates tested after five passages are given in the parentheses. ND – not resistant isolates initially detected.

A high proportion (83.3 -100.0 %) of *E.coli* isolated from settled sewage, final treated effluent and surface waters both up- and down-stream from the final effluent discharge point, initially identified as resistant to amoxicillin (according to both ECOff and CBP values) maintained their resistance. Similarly, all four *E.coli* isolates (100.0 %) identified as resistant to ciprofloxacin using resistant CBP values (R > 1 mg/L) retained their resistance.

A higher proportion (83.3 %) of *E.coli* isolated from settled sewage and final treated effluent classified with acquired ciprofloxacin resistance (using ECOff values) maintained their resistance compared with the proportion of *E.coli* isolated from surface water down-stream of the discharge point that maintained their resistance (57.1 %). However, there is not enough evidence (2-proportion test; P > 0.05) to suggest that the proportion of *E.coli* isolates that have maintained their resistance are statistically different. All *E.coli* isolates initially identified as resistant to cefpodoxime (using CBP and ECOff values) maintained their

resistance level. In the surface water sample collected up-stream of the effluent discharge point, no ciprofloxacin or cefpodoxime resistant strains were detected and hence no sub-culture experiments performed.

5.3.4 Antibiotic resistance in *E.faecium* from environmental waters

5.3.4.1 E.faecium MIC determination

The amoxicillin, ciprofloxacin and clarithromycin minimum inhibitory concentrations (MICs) measured for *E.faecium* isolated from settled sewage (n = 32), final treated effluent (n =38) and surface waters both up- (n = 35) and down-stream (n = 23) of the final effluent discharge point are presented in Table 5-17. Additionally, vancomycin MIC values were measured for 32 (settled sewage), 34 (final effluent), 19 (up-stream) and 25 (down-stream) isolates of *E.faecium* from the same samples.

The amoxicillin, ciprofloxacin and clarithromycin MIC values measured span the concentrations that define sensitive (according to clinical breakpoint values) and wild type strains (according to epidemiological cut of values) up to concentrations greater than those used to define resistant and non-wild type strains. The vancomycin MIC values measured for *E.faecium* indicate all isolates tested in this study were vancomycin sensitive and wild type.

The concentrations of amoxicillin (0.38 - 0.5 mg/L), ciprofloxacin (2 - 4 mg/L), clarithromycin (2 - 6) and vancomycin (1 - 2 mg/L) that inhibited 50 % of the *E.faecium* isolates (MIC_{50}) tested were similar (within a two-fold dilution) for all sampling points (Table 5-18).

Table 5-17: Amoxicillin, ciprofloxacin, clarithromycin and vancomycin minimum inhibitory concentrations (mg/L) for *E.faecium* isolated from wastewaters and surface waters

Antibiotic	Sample point	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Amoxicillin	Settled sewage (n = 32)	0.064-256	0.38	64
	Final effluent (n = 38)	0.032-256	0.5	12
	Up-stream (n = 23)	0.094-4	0.38	0.75
	Down-stream (n = 35)	0.064-32	0.38	1.0
Ciprofloxacin	Settled sewage (n = 32)	0.19-32	2	8
	Final effluent (n = 39)	0.25-32	2	32
	Up-stream (n = 23)	0.38-32	4	32
	Down-stream (n = 35)	0.25-32	2	8
Clarithromycin	Settled sewage (n = 32)	0.19-64	6	12
	Final effluent (n = 39)	0.25-256	4	256
	Up-stream (n = 23)	0.25-256	2	12
	Down-stream (n = 35)	0.25-24	6	12
Vancomycin	Settled sewage (n = 32)	0.38-4	2	3
	Final effluent (n = 34)	0.19-4	1	2
	Up-stream (n = 19)	0.5-4	1	2
	Down-stream (n = 25)	0.38-4	1	3

MIC_{50} is equivalent to the concentration of antibiotic that inhibits 50% of *E.faecium*; MIC_{90} is equivalent to the concentration of antibiotic that inhibits 90% of *E.faecium*.

The concentration of amoxicillin (0.75 – 1 mg/L) that inhibited 90 % of the *E .faecium* tested (MIC_{90}) was lower for surface waters up- and down-stream of the treated effluent discharge point compared to the settled sewage (64 mg/L) and final treated effluent (12 mg/L) although statistically not significantly different (Mann-Whitney; P > 0.05). The concentrations of ciprofloxacin (8 - 32 mg/L), clarithromycin (12 – 256 mg/L) and vancomycin that inhibited 90 % of *E.faecium* isolates were above the clinical breakpoint values given in Table 5-4, for all sample points.

5.3.4.2 Distribution of the antibiotic minimum inhibitory concentration values determined for E.faecium taken from wastewater and receiving surface water

The distribution of amoxicillin MIC values measured for *E.faecium* isolates in settled sewage, final treated effluent and surface waters both up- and down-stream of the final effluent discharge point are presented in Figure 5-8. It can be seen that *E.faecium* resistant to amoxicillin according to the clinical breakpoint value (R > 8 mg/L) were present at each sampling point apart from the surface waters up-stream of the effluent discharge point.

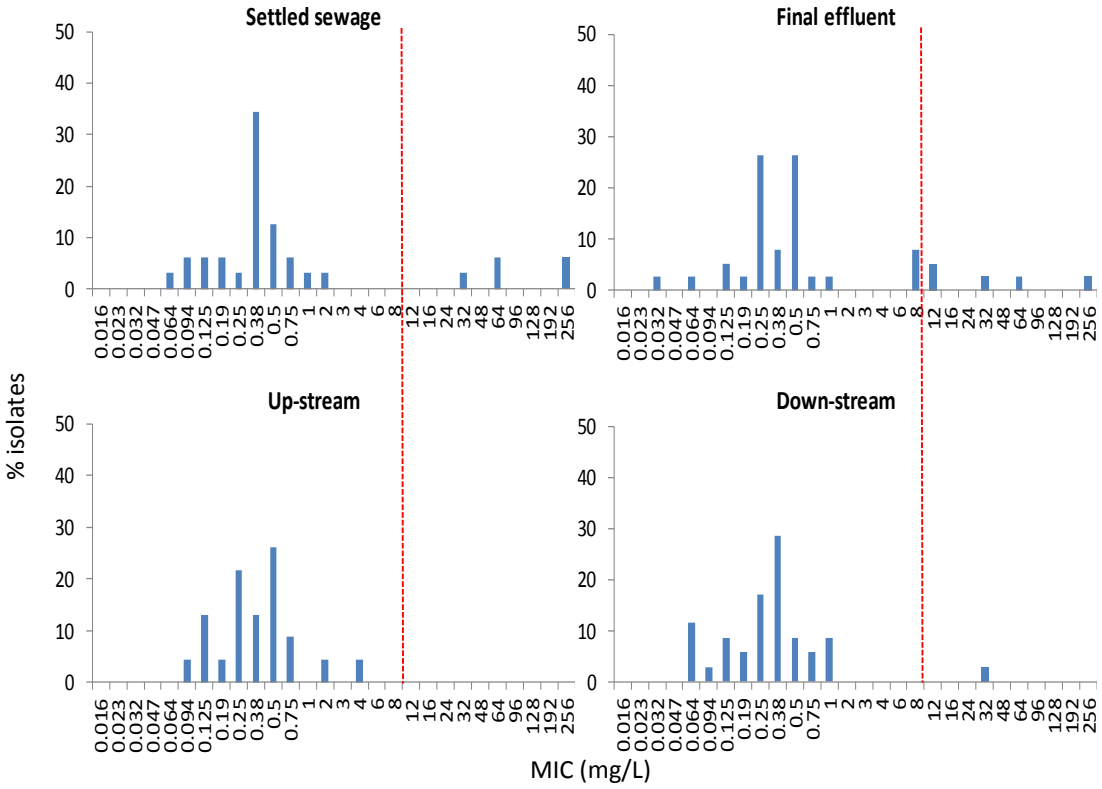


Figure 5-8: Distribution of amoxicillin MIC values measured for *E.faecium* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The red dashed line represents the clinical resistance breakpoint (R > 8 mg/L). There is no epidemiological cut off value reported for *E.faecium*/ amoxicillin.

The distribution of ciprofloxacin MIC values measured for *E.faecium* isolated from all sampling points is presented in Figure 5-9. *E.faecium* resistant to ciprofloxacin (according to both epidemiological cut off values and clinical breakpoints, which are identical at 4 mg/L, were identified at all sampling points (settled sewage, final treated effluent and surface water both up- and down-stream relative to the effluent discharge point).

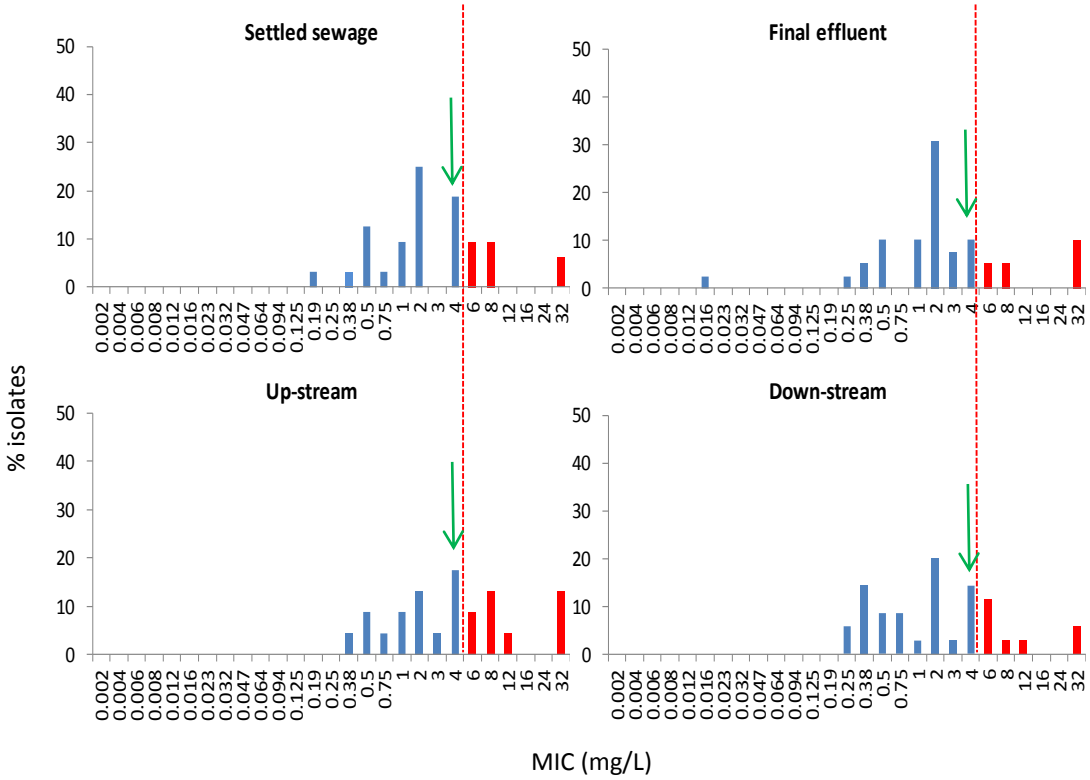


Figure 5-9: Distribution of ciprofloxacin MIC values measured for *E.faecium* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The green arrow represents the epidemiological cut-off value (WT ≤ 4.0 mg/L). The blue bars represent wild type *E.faecium* (wild type) and the red bars represent non-wild type *E.faecium* (acquired resistance). The red dashed line represents the clinical breakpoint value (R > 4.0 mg/L)

The distributions of clarithromycin MIC values measured for the *E.faecium* strains isolated from all sampling points are presented in Figure 5-10 and demonstrate that *E.faecium* with acquired clarithromycin resistance are present at each sampling point. The changes in clarithromycin resistance rates in *E.faecium* were assessed only against the epidemiological cut of value as there are no clinical breakpoints reported by EUCAST (2012).

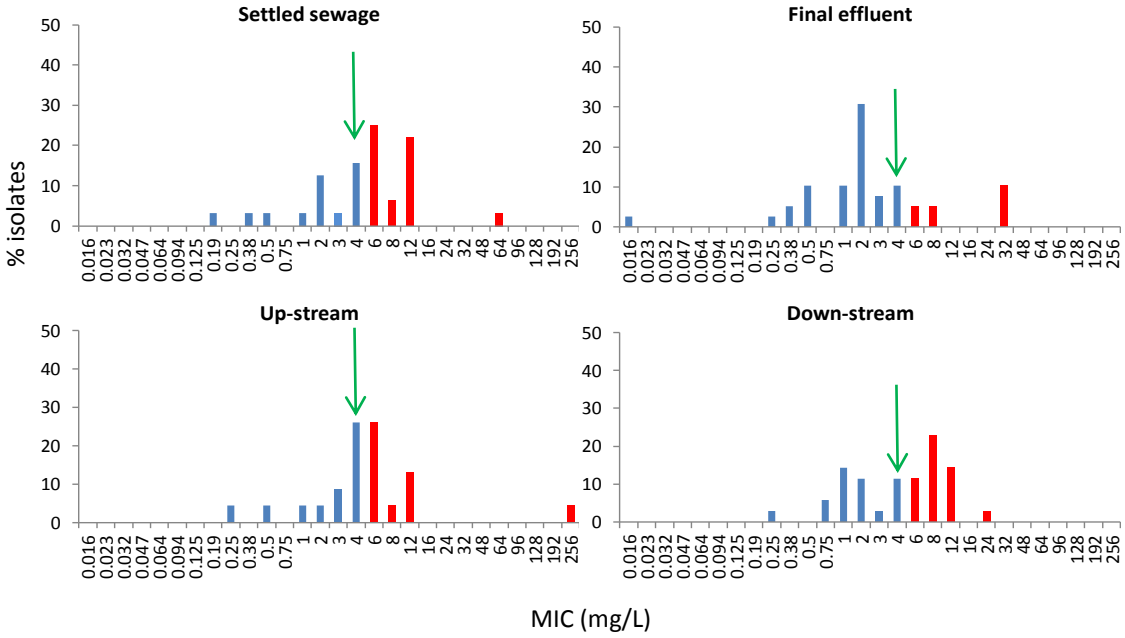


Figure 5-10: Distribution of clarithromycin MIC values measured for *E.faecium* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The green arrow represents the epidemiological cut-off value (wild type ≤ 4 mg/L). The blue bars represent wild type *E.faecium* (wild type) and the red bars represent non-wild type *E.faecium* (acquired resistance). There are currently no clinical breakpoints for *E.faecium*/ clarithromycin reported by EUCAST.

Only vancomycin sensitive *E.faecium* strains were detected in the settled sewage, final treated effluent and receiving surface water both up- and down-stream from the final effluent discharge point as shown in Figure 5-11.

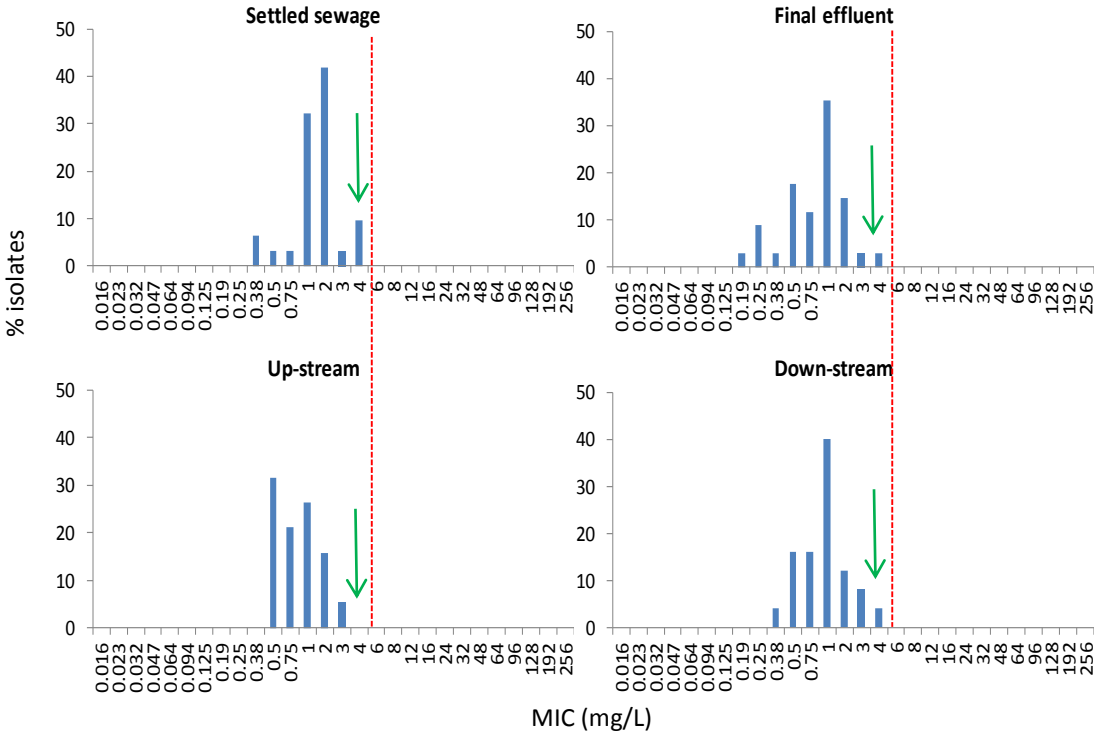


Figure 5-11: Distribution of vancomycin MIC values measured for *E.faecium* isolated from settled sewage, final effluent and surface waters up- and down-stream of the effluent discharge point. The green arrow represents the epidemiological cut-off value (WT ≤ 4 mg/L). The red dashed line represents the clinical resistance breakpoint (R > 4 mg/L).

5.3.4.3 Proportion of E.faecium resistant to antibiotics in wastewaters and surface water

Overall from a total 129 *E.faecium* isolates obtained from all sampling points, resistance to clarithromycin (51.9 %) was more prevalent in *E.faecium* than was the case for ciprofloxacin (25.6 %) or amoxicillin (8.5 %). No vancomycin resistant sensitive or non-wild type strains of *E.faecium* strains were detected in any of the monitored water samples (Table 5-18).

Table 5-18: The proportion (%) of *E.faecium* isolates resistant (according to ECOFF and CBP values) to amoxicillin, ciprofloxacin, clarithromycin and vancomycin.

	% E.faecium				
	Settled Sewage (n = 32)	Final effluent (n = 39)	Down-stream (n = 35)	Up-stream (n 23)	[b] % of total isolates (n =129)
Amoxicillin ECOff	No ECOff	No ECOff	No ECOff	No ECOff	No ECOff
[a] CBP	15.6 (5.3 -32.8)	13.2 (4.4 – 28.1)	2.9 (0.1 – 28.1)	ND	8.5 (4.3 – 14.7)
Ciprofloxacin ECOff	25.0 (11.5– 43.4)	20.5 (9.3 – 36.5)	22.9 (10.4 – 40.1)	39.1 (19.7 – 61.5)	25.6 (18.3 -34.0)
CBP	25.0 (11.5– 43.4)	20.5 (9.3 – 36.5)	22.9 (10.4 – 40.1)	39.1 (19.7 – 61.5)	25.6 (18.3 -34.0)
Clarithromycin ECOff	56.3 (37.7 – 73.6)	51.3 (34.8 – 67.6)	51.3 (34.8 – 67.6)	47.8 (26.8 – 69.4)	51.9 (43.0 – 60.8)
CBP	No CBP	No CBP	No CBP	No CBP	No CBP
Vancomycin ECOff	ND	ND	ND	ND	ND
CBP	ND	ND	ND	ND	ND
[c] Multi antibiotics ECOff	6.3 (0.8 -20.8)	12.8 4.3 - 27.4)	14.3 (4.8–30.3)	13.0 (2.8 -33.6)	11.6 (6.7 -18.5)
CBP	12.5 (3.5 – 28.9)	10.3 (2.9 – 24.2)	2.9 (0.1 – 15.0)	None	7.0 (3.2 -12.8)

1-proportion 95 % confidence intervals are given in the parentheses. ND = none detected. No CBP or No ECOff = No clinical breakpoint or epidemiological cut off value. [a] significantly different proportions (2-proportions test; $P < 0.05$) amongst sampling points. [b] overall % of total isolates from all sampling points (n = 129). [c] Multi antibiotics = % of total isolates with acquired resistance (according to ECOff values – not including amoxicillin) or resistant (according to CBP values – not including clarithromycin) to more than one of the antibiotics tested. None – no isolates identified with resistance to multiple antibiotics.

Elevated levels of *E.faecium* resistant to amoxicillin were observed in the settled sewage (15.6 %) and final treated effluent (13.2 %) compared to the surface waters (2 proportions test; $P < 0.01$). However, the proportion of *E.faecium* resistant to clarithromycin and ciprofloxacin was more consistent in all collected water samples (Table 5-18). According to the epidemiological cut off values for clarithromycin, ciprofloxacin and vancomycin, 11.6 %

(n = 15) of the total *E.faecium* isolated (n = 129) from the water samples demonstrated acquired resistance to more than of one the tested antibiotics (excluding amoxicillin as there is no ECOff value currently determined). In addition, It was observed that the proportion of *E.faecium* with acquired resistance to more than one of the tested antibiotics were similar (2-proportions test; P > 0.05) between all sampling points (Table 5-18). When using the clinical breakpoint values, a lower percentage of *E.faecium* (n =1; 2.9%) with resistance to more than one antibiotic (excluding clarithromycin due to no CBP value) was observed in surface water down-stream from the WWTP treated effluent discharge point compared to the settled sewage (n =4; 12.5%) and final treated effluent (n = 4; 10.3%). No isolates with multi resistance to more than of the tested antibiotics (according to CBP values) were observed up-stream.

5.3.4.4 Maintenance of resistance in E.faecium

The proportion of *E.faecium* resistant to amoxicillin, ciprofloxacin and clarithromycin (according to ECOff and CBP values) that maintained their resistance following the repeated (10 times) sub-culture on antibiotic free nutrient agar are presented in Table 5-19. Interestingly, following repeated sub-culturing, all the *E.faecium* isolates originally identified as resistant maintained their resistance.

Table 5-19: Proportion (%) of resistant *E.faecium* isolates maintaining resistance, following repeated sub-culture

Resistance to	% maintained resistance			
	Settled sewage	Final treated effluent	Down-stream	Up-stream
[a] Amoxicillin	100.0 (5)	100.0 (5)	100.0 (1)	ND
[b] Ciprofloxacin	100.0 (7)	100.0 (8)	100.0 (8)	100.0 (6)
[c] Clarithromycin	100.0 (18.0)	100.0 (15)	100.0 (18)	100.0 (10)
[b] vancomycin	ND	ND	ND	ND

[a] according to CBP values (R > 8 mg/L), [b] to CBP and ECOff values (both 4 mg/L), [c] to the ECOff value (WT $\leq$ 4 mg/L). Total number of isolates tested after five passages are given in the parentheses. ND – not resistant isolates initially detected.

5.4 Discussion

5.4.1 Indicator bacteria in wastewater and surface water

Total heterotrophic bacteria represent a pool of cultivable and viable bacteria that could acquire antibiotic resistance or spread antibiotic resistance. In this study, the concentrations of heterotrophic bacteria in settled sewage and final treated effluent (Table 5-6) are in agreement (~ 9.0 $\log_{10}$ and 8.0 $\log_{10}$ CFU/100 mL heterotrophic bacteria in raw and treated wastewater respectively) with those previously reported for urban wastewaters (Ferreira Da Silva et al., 2006). In addition, the concentrations of total coliforms, *E.coli* and enterococci detected in the settled sewage and final treated effluent (Table 5-6) are similar to reports for raw wastewaters (~ 7.0, 6.0 and 6.0 $\log_{10}$ CFU/100 mL for total coliforms, *E.coli* and enterococci respectively) and treated wastewaters (~ 6.0, 4.0 and 3.0 $\log_{10}$ CFU/100 mL for total coliforms, *E.coli* and enterococci respectively) taken from urban wastewater treatment

plants elsewhere in Europe (Blanch et al., 2003; Silva et al., 2006; Silva et al., 2007; Servais et al., 2009). Although the wastewater treatment process significantly reduced the concentration of indicator bacteria (> 97 %), high numbers were still prevalent in the final treated effluents. This is a concern as the abundant levels of nutrients and oxygenated conditions combined with high concentrations of bacteria and low concentrations of antibiotics in activated sludge treatment tanks have the potential to stimulate bacteria in the dissemination of resistance genes on mobile genetic elements (Kummerer, 2004).

In this study, the bacterial concentrations measured in the receiving surface water (down-stream) were similar (within 0.1 – 0.4 logs) to the concentrations observed in the final treated effluent (Table 5-6). This was expected as there is a long history of water quality issues with the river water sampled (e.g. high sediment accumulation and water abstraction). The concentrations of indicator bacteria within the surface water up-stream from treated effluent discharges were typically one order of magnitude less than those observed in the final effluent. Heterotrophic bacteria and faecal indicators can often be present at levels two or three orders of magnitude greater in surface waters down-stream of wastewater effluent discharge points compared to up-stream (Maier et al., 2009) supported by the presence of elevated amounts of organic matter and nutrients in treated effluent discharges. Studies by Goni-Urriza et al. (2000) have reported significant increases of coliforms and *E.coli* in surface waters receiving treated effluent (*E.coli* concentration ~ 6.0 $\log_{10}$ CFU/100 mL) compared to surface water up-stream (*E.coli* concentration ~ 3.0 $\log_{10}$ CFU/100 mL) from treated effluent discharge points, whilst a wide range of enterococci concentrations (1.5 – 3.2 $\log_{10}$ CFU/100 mL) in a variety of surface waters down-stream from different treated effluent discharge points were observed by Blanch et al. (2003).

5.4.1.1 Distribution of E.faecium in wastewater and surface water

E.faecium was the most prevalent of the enterococci species in the settled sewage, final treated effluent and receiving surface water (down-stream) (Figure 5-4). Wastewater treatment processes, did not influence the proportion of *E.faecium* and therefore the proportions of *E.faecium* in the settled sewage (73.1 %) and final treated effluent (74.6 %) were similar. This is in contrast to the results reported by Ferreira Da Silva et al. (2006), who identified *E.hirae* as the most abundant species in urban wastewaters and observed an increase in the proportion of *E.faecium* following activated sludge treatment. Some studies have found a higher prevalence of *E.hirae* in environmental waters associated with pigs and cattle (Kuhn et al., 2003). Interestingly Blanch et al. (2003) reported that *E.faecium* was the most prevalent species in the wastewaters and receiving surface waters in Spain and the UK whilst contrastingly in Sweden, *E.faecalis* was the most prevalent species. The different proportions of *E.faecium*, *E.faecalis* and *E.hirae* in wastewaters, reported in other studies, could be due to differences in the catchment area diet, seasonal differences or the type of wastewater treatment employed (Layton et al., 2009 and Blanch et al., 2003).

The similar proportions of *E.faecium* in the final treated effluent and receiving surface waters demonstrate that the treated wastewater effluent discharges impact the receiving surface water. This is because *E.faecalis* and *E.faecium* are typically the most prevalent of species in human faeces and thus the most common species in urban wastewater (Layton et al., 2009). A higher predominance of *E.faecium* within wastewaters (91.0 – 96.0 %) and receiving surface waters (89.0 %) compared to other enterococci species was also found by Leclercq et al. (2007). Species such as *E.casseliflavus* are more associated with plants

(Layton et al., 2009) and *E. durans* and *E.hirae* are considered to be mainly of animal origin (Klein, 2003). Therefore, the lower prevalence of *E.faecium* (38.5 %) and the greater diversity in species of the enterococci genera observed in surface waters up-stream from the effluent discharge point compared to the other monitored samples was expected. Due to the higher predominance of *E.faecium* in the collected water samples indicating human activity, this species was selected as an ideal candidate for monitoring the transfer of antibiotic resistance from wastewater to surface waters.

5.4.2 Interpretive criteria for assessing antibiotic susceptibility in environmental bacteria

Currently, there are two different types of interpretive criteria that are available for interpreting in vitro antibiotic susceptibility tests. These are clinical breakpoint values (CBP values) and epidemiological cut-off values (ECOff values) as introduced in Chapter 3, section 3.4.1. Clinical breakpoints are useful to determine if a bacterium responds to therapy. Whereas, epidemiological cut off values help define bacteria that possess any kind of resistance mechanisms or resistance genes (Walsh, 2013). Although CBP and ECOff values are determined by different approaches they can be identical for some bacteria/drug combinations. For example the CBP and ECOff values are identical for the combination *E.faecium*/ vancomycin. However, there are exceptions when the ECOff value is either lower (*E.coli*/ ciprofloxacin) or greater (*E.coli*/ cefpodoxime) than the CBP value and hence different conclusions can be drawn depending on the interpretive criteria used.

In this study, *E.coli* resistance levels to ciprofloxacin were underestimated when the resistant clinical breakpoint value was used (1.8 %) compared to the epidemiological cut off value (9.2 %). Conversely, cefpodoxime resistance levels in *E.coli* taken from environmental

waters (4.3 %) were overestimated using the current cefpodoxime resistant clinical breakpoint value compared to the epidemiological cut off value (1.6 %) as presented in Table 5-15. Furthermore, when using clinical breakpoint values, no *E.faecium* isolates (taken from all samples) were identified with resistance to multiple antibiotics (from those tested). However, when using the epidemiological cut off value, 13 % of the *E.faecium* isolates were identified to have acquired resistance to multiple antibiotics (Table 5-18). The comparison of the MIC valves with both harmonised clinical breakpoint and epidemiological cut off values from the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2000) has demonstrated that there is an urgent need for the standardisation of the interpretive criteria used for assessing antibiotic susceptibility data. In addition, this data demonstrates that the monitoring of antibiotic minimum inhibitory concentration values within a species and the use of epidemiological cut of values for interpretation is the most appropriate method for detecting subtle changes in resistance.

Schwarz et al. (2010) have suggested that antibiotic susceptibility surveillance publications should include the distribution of minimum inhibitory concentration values for each bacterial species/drug combination, so that readers may use the reported data in the right context. This is also important for bacterial species/antibiotic combinations that have no defined ECOff or CBP values. For example, there is no ECOff value for amoxicillin susceptibility in *E.faecium* and no CBP value for clarithromycin susceptibility in *E.faecium*

Problems associated with surveillance programmes using different antibiotic susceptibility interpretative criteria were illustrated in a report by Silley et al. (2011). When applying the clinical breakpoint value (> 2 mg/L) to the surveillance of ciprofloxacin susceptibility in

salmonella taken from animals (in the Netherlands in 2004), 0.3 % were found to be resistant. The surveillance carried out the following year showed elevated resistance levels in *salmonella* (10.1 %). However the rise is misleading as the ECOff value (> 0.06 mg/L) was used in this case. This demonstrates that the term resistance although frequently used is not clearly understood and emphasises that the standardisation and harmonisation of values used to define resistance are imperative for effective surveillance of antibiotic resistance development. In addition, a single standardised approach to assessing antibiotic susceptibility is crucial for effective risk assessment and when the comparison of resistance from different sources (e.g. food, environment and humans) is required.

5.4.3 The antibiotic susceptibility of *E.faecium* and *E.coli* in environmental waters

The results obtained show that *E.coli* resistant (according to ECOff and CBP values) to amoxicillin, and to ciprofloxacin and cefpodoxime and *E.faecium* resistant to amoxicillin, ciprofloxacin and clarithromycin were present in environmental waters (settled sewage, treated effluent and surface waters). In addition, isolates with resistance to more than one antibiotic were also present.

The prescription levels estimated for England in 2011 (Chapter 4) for amoxicillin (168 tonnes a.i), ciprofloxacin (7 tonnes a.i) and cefpodoxime (0.002 tonnes a.i) correlate with the levels of resistance (according to ECOff values) to amoxicillin (20.1 %), ciprofloxacin (9.2 %) and cefpodoxime (1.6 %) observed in *E.coli*. By comparing the levels of resistance in *E.coli* to prescription levels it can be hypothesised that clinical use of these substances has influenced the positive selection of resistant strains. Similarly, the high prescription quantities of clarithromycin (12 tonnes a.i) and ciprofloxacin (7 tonnes a.i) and their

presence in the active form in environmental waters (although at sub inhibitory concentrations) may account for the high proportion of *E.faecium* resistant to clarithromycin (51.9 %) and ciprofloxacin (25.6 %) in the wastewater and surface water samples. In contrast, a low prevalence of *E.faecium* resistance to amoxicillin was observed (8.5 %). despite the elevated use of amoxicillin in medicine and the high prevalence of penicillin (including amoxicillin) resistance observed in *E.faecium* through clinical surveillance (93.1 % in 2012) in England (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2014a). However, a lower prevalence of resistance in environmental samples compared to clinical samples would be expected. The absence *of E.faecium* strains resistant to vancomycin was also expected (Figure 5-11) and may reflect the low prescription levels in England each year (0.03 tonnes a.i) and the decrease in the prevalence of vancomycin resistant *E.faecium* observed through clinical surveillance (33.0 -13.3 %) between 2005 -2012 (European Centre for Disease Prevention and Control - Antimicrobial resistance interactive database (EARS-net), 2014b). This is also consistent with the report from Martins da Costa et al. (2007) who found very low proportions of enterococci in wastewaters resistant to vancomycin (0.6 %).

5.4.3.1 The antibiotic susceptibility of E.coli and E.faecium in wastewater

Whether resistance develops throughout the wastewater treatment process is still currently under discussion. Wastewater treatment plants are a reservoir of antibiotics (Watkinson et al., 2007), antibiotic resistant bacteria (Faria et al., 2009) and antibiotic resistance genes (Munir et al., 2011; Szczepanowski et al., 2009). Therefore, in combination with the presence of high nutrient and oxygen levels, it is believed that WWTPs provide desirable

conditions for the dissemination of antibiotic resistance (Novo et al., 2010; Luczkiewicz et al., 2010).

There are studies that have reported increases in the proportion of resistant bacteria throughout the wastewater treatment process. Silva et al. (2007) found comparatively higher levels of amoxicillin (28.0 and 34.8 % before and after activated sludge treatment) and ciprofloxacin (2.5 and 9.7 % before and after activated sludge treatment) resistant *E.coli* following activated sludge treatment and Silva et al. (2006) reported that wastewater treatment increased enterococci resistance levels to ciprofloxacin from 9 to 25 %. Conversely, Guardabassi et al. (2002) found that the wastewater treatment process significantly reduced ($P < 0.05$) the levels of antibiotic resistant bacteria (*Acinetobacter*). In addition, a reduction in the levels of antibiotic resistant bacteria and antibiotic resistant genes with wastewater treatment was observed by Munir et al. (2011).

Interestingly, the results from this study demonstrate that wastewater treatment did not positively or negatively select for antibiotic resistant *E.coli* and *E.faecium*. Therefore this data does not support the hypothesis that wastewater treatment processes serve as potential hotspots for the dissemination of resistance genes or elements (Guardabassi et al., 2002). However, despite the large reduction of the faecal bacteria with activated sludge treatment (Table 5-7), the wastewater treatment did not reduce the proportion *E.coli* resistant to amoxicillin, ciprofloxacin and cefpodoxime or the proportion of *E.faecium* resistant to amoxicillin, ciprofloxacin and clarithromycin. This suggests that the presence of low concentrations of antibiotics detected in wastewater (presented in Chapter 4) may serve as a selective pressure to maintain the proportion of resistance among faecal bacteria. This is also

in agreement with the work reported by Galvin et al. (2010) and Koczura et al. (2012), who found that the levels of antibiotic resistant *E.coli* were not eliminated by wastewater treatment. Similarly, Leclercq et al. (2007) observed the level of resistance within enterococci were not affected by the treatment process.

There are a number of factors that may influence the fate of antibiotic resistant bacteria in WWTPs including the type of wastewater treatment and treatment plant operation (Bouki et al., 2013). Activated sludge treatment processes with enhanced sludge retention times may correspond to an enhancement in the levels of antibiotic resistant bacteria (Luczkiewicz et al., 2010). Longer hydraulic retention times or sludge retention times may give rise to a more diverse biomass that may favour horizontal gene transfer processes and therefore the dissemination of resistance (Novo et al., 2010; Luczkiewicz et al., 2010). Whilst differences in the initial composition of sewage may account for the varying levels of antibiotic resistant bacteria (Guardabassi et al., 2002). Munir et al. (2011) found the level of the reduction of antibiotic resistant bacteria levels varied between different types of treatment facilities. Significantly higher reductions of antibiotic resistant bacteria and antibiotic resistant genes were observed in a membrane biological reactor facility compared to treatment facilities employing activated sludge treatment. However, no significant differences in the levels of antibiotic resistant bacteria were found between pre- and post-disinfected wastewaters. Seasonal differences and climate differences may also impact on the dissemination of antibiotic resistance in wastewater treatment facilities. Novo et al. (2013) reported that the levels of antibiotic resistant bacteria in wastewaters differed depending on the season. It was found that the wastewater treatment process significantly increased the levels of amoxicillin and ciprofloxacin resistance in autumn but not in the spring.

Although reports do vary, collectively they indicate that wastewater treatment plants do not completely eliminate antibiotic resistant faecal bacteria from wastewater. Consequently, treated effluents may be a constant source of antibiotic resistant faecal bacteria to receiving water thus contributing to the spread of resistant bacteria in environmental waters.

5.4.3.2 The antibiotic susceptibility of E.faecium and E.coli in surface waters

In order to study the impact of the final treated effluent on the antibiotic resistance of a receiving surface water, the resistance levels of *E.coli* and *E.faecium* up- and down-stream of the WWTP final effluent discharge point were also determined.

High proportions of *E.coli* and *E.faecium* with acquired antibiotic resistance were identified in the wastewater treated effluent demonstrating WWTPs do not completely eliminate antibiotic resistant bacteria (Table 5-15 and Table 5-18). Consequently they are discharged to the environment. It was expected that the levels of faecal bacteria in the final treated effluent discharges would be diluted when discharged to the river water. However, the levels in the final treated effluent and surface water down-stream from the WWTP treated effluent discharge point were similar (Table 5-6). Correspondingly, the levels of *E.coli* with acquired resistance to amoxicillin and ciprofloxacin (Table 5-15) and the levels *E.faecium* (Table 5-18) with acquired resistance to ciprofloxacin and clarithromycin were similar (2-proportions test; $P > 0.05$) between the final treated effluent and surface water down-stream of the discharge point.

Significant increases in the proportion of *E.coli* resistant to antibiotics in surface waters down-stream of treated effluent discharges were observed compared to up-stream by Koczura et al. (2012). Similarly, Zhang et al. (2009) found higher numbers of resistant

bacteria in river water samples taken down-stream from treated effluent discharges than in up-stream river water samples. Therefore, it was hypothesised that the proportion of acquired resistance in *E.coli*, taken from surface waters down-stream of the treated effluent discharge point, would be elevated compared to up-stream.

In this study, the proportion of *E.coli* with acquired resistance to amoxicillin and ciprofloxacin was more elevated in the surface water down-stream compared to up-stream relative to the WWTP treated effluent discharge point. This indicates that the discharges of treated effluent influence the proportion of resistance in *E.coli* in the receiving surface water compared to surface water up-stream from effluent discharges. The lower prevalence of resistance observed up-stream probably correspond to problems with leakages from sewer misconnections which may act as a source of antibiotic resistant bacteria further up-stream.

The presence of *E.coli* and *E.faecium* with acquired resistance to antibiotics in surface waters indicates that the bacterial strains with acquired resistance are not at any significant survival disadvantage in environmental waters. Studies by (Flint, 1987; Caldwell et al., 1989) support this conclusion as they have shown resistance plasmids are stable within *E.coli* whilst under survival conditions in river water. This is supported further by reports from Korezeniewska (2013), who identified large proportions (8 – 32 %) of *E.coli* with resistance to antibiotics (e.g. penicillins) in surface waters receiving urban wastewater treated effluent discharges and by Zurfluh (2013) who identified a high proportion (36 %) of *E.coli* taken from a variety of river water samples carrying plasmid mediated resistance mechanisms to β lactam antibiotics. In addition, these findings are consistent with the enterococci

fluoroquinolone resistant levels observed in surface waters by Junco et al. (2001) and the macrolide resistance levels identified in Sweden and Spain by Blanch et al. (2003). Additionally, these data indicate that the presence of low levels of antibiotics present in the treated effluents and receiving surface waters may select for resistance within the faecal bacteria population. However, studies such as those reported by Muela et al. (1994) found horizontal gene transfer processes within the *E.coli* population were inhibited in surface water conditions (e.g. low nutrient levels).

Previous reports have demonstrated ciprofloxacin and clarithromycin resistance is widespread in enterococci from humans as well as other sources. Studies carried out by Barry et al. (1984) found that shortly after the introduction of ciprofloxacin (1983), approximately 50 % of clinical *E.faecium* isolates were resistant. Similarly, Kuhn et al. (2000) found macrolide resistance was widespread in enterococci from humans, pigs and broilers and Eliopoulos (2007) identified that macrolide resistance was very common among clinical isolates of *E.faecium*. Therefore it was expected that resistant *E. faecium* isolates would be identified at all sampling points including the surface water up-stream of the treated effluent discharge point. The proportions of *E.faecium* with acquired resistance to ciprofloxacin and clarithromycin were similar between the surface waters collected up- and down-stream of the treated effluent discharge point and also similar to the wastewater samples (presented in Table 5-18), highlighting that treated wastewater effluent is not the only source of resistant *E. faecium* isolates to surface waters.

Conversely, a decrease in the proportion of amoxicillin resistant *E.faecium* was observed in the receiving surface water compared to the wastewater samples. In the surface water

down-stream from the WWTP treated effluent discharge point, only 2.9 % of *E.faecium* isolates were identified as amoxicillin resistant. Whereas, no amoxicillin resistant *E.faecium* resistant isolates were identified in the water samples collected from up-stream (Figure 5-8). This may be related to the poor survival of amoxicillin resistant strains in surface water or more likely due to the dilution of the resistant strains with more sensitive enterococci counterparts (Leclercq et al., 2007). It has been reported that enterococci penicillin (including amoxicillin) resistance is widespread in human clinical isolates but not in animals, poultry and food (Mannu et al., 2003, Martins da Costa et al., 2007). Considering that the enterococci present in surface waters will be from a variety of sources (animals and plants) in addition to the treated effluent discharges, it was not surprising that the proportion of *E.faecium* resistant to amoxicillin was lower in the surface water compared to wastewater.

Overall, the elevated proportion of resistant *E.faecium* and *E.coli* in surface waters is of concern. Appropriate measures need to be taken in order to monitor and assess the anthropogenic contribution of antibiotic resistance to the environment. New strategies for the treatment of wastewater or more stringent treatments need to be assessed to reduce the proportion of resistant bacteria present in surface waters.

5.5 Summary

E.coli and *E.faecium* were isolated from settled sewage, treated effluent and surface water both up- and down-stream of an effluent discharge point using culturable methods for subsequent quantitative antibiotic susceptibility testing.

- Due to the difficulty of selectively detecting *Staphylococcus aureus* and *Pseudomonas aeruginosa* in wastewater and surface water samples in high proportions using recommended culturable methods it was decided not to investigate the antibiotic resistance profiles of these bacterial species in environmental waters. In addition, as they are not exclusively of faecal origin (Environment Agency, 2000) but are commonly found in soil and on plants they are not necessarily ideal indicator organisms to use to assess the impact of urban treated effluent on receiving surface waters.
- *E.coli* was easily selected and differentiated from other enterobacteriaceae using β-galactosidase/β glucuronidase chromogenic media with a high *E.coli* confirmation rate (91.6 %) that is comparable to reports from other studies (Alonso et al., 1996; Wohlsen, 2011). The identification of the presumptive coliform colonies (Table 5-8) indicated a diverse range of species from the coliform group which are all of faecal origin (*Citrobacter*, *Klebsiella* and *Enterobacter*) and therefore antibiotic susceptibility tests were carried out only for *E.coli* and not other coliform bacteria.
- The use of Slanetz and Bartley media for the detection and enumeration of enterococci can facilitate the growth of other bacteria physiologically similar to enterococci (e.g. *Lactococcus* spp, *Leuconostoc* spp and *Aerococcus* spp) and does not enable the distinction between different *Enterococci* species (Swenson et al., 1990). However, with additional identification using MALDI-TOF-MS analysis it was demonstrated that *E.faecium* were present in high enough proportions in the wastewater and surface water to study antibiotic resistance profiles.

- The proportion of *E.coli* from environmental waters resistant to ciprofloxacin was underestimated when minimum inhibitory concentrations were interpreted using clinical breakpoints. Contrastingly, the levels of cefpodoxime resistance in *E.coli* were overestimated when using the resistant clinical breakpoint.
- Different antibiotic resistance patterns were observed between *E.coli* and *E.faecium* from settled sewage, treated effluent and surface water. Overall it was found that antibiotic resistance is more prevalent in *E.faecium* than *E.coli*. However, elevated levels of *E.coli* were more resistant to amoxicillin compared to *E.faecium*.
- Wastewater treatment did not significantly change the proportion of *E.coli* resistant to amoxicillin, ciprofloxacin and cefpodoxime or the proportion of *E.faecium* resistance to amoxicillin, ciprofloxacin and clarithromycin. Only vancomycin sensitive *E.faecium* strains were detected in the wastewater.
- According to epidemiological cut off values, elevated levels of *E.coli* resistant to amoxicillin and ciprofloxacin and *E.faecium* resistant to amoxicillin were observed in the surface water down-stream of the treated effluent discharge point compared to the surface water up-stream, indicating treated effluent discharges influence the levels of antibiotic resistant bacteria in receiving surface water. However, similar clarithromycin and ciprofloxacin resistance levels for *E.faecium* were observed at all sampling points.

6 *The prevalence of E.coli with* resistance to ciprofloxacin within constructed microcosms.

6.1 Introduction

There are now increasing reports of the presence of antibiotic residues and antibiotic resistant bacteria in wastewaters and river waters (Moore et al., 2010; Lacey et al., 2008; Senta et al., 2008; Guardabassi et al., 1998). However, very few studies have examined the impact that antibiotic residues have on the development of resistance in these environments and particularly in surface waters. Studies by Zhang et al., (2009) and Engemann et al. (2008) are two examples. There is increasing evidence that sub-therapeutic levels of antibiotics can promote the dissemination of antibiotic resistance in bacteria in clinical (Gullberg et al., 2011) and animal husbandry environments (Gellin et al., 1989). Therefore it is possible that the antibiotics episodically released into river waters within treated effluent discharges, although at sub-therapeutic levels, may exert the necessary pressure to support the development and spread of resistant bacteria.

In Chapter 5, it was observed that a greater proportion of ciprofloxacin resistant *Escherichia coli* (according to epidemiological cut off values) were present in the surface water down-stream from the wastewater treated effluent discharge point compared to up-stream (Section 5.3.4.3). However, it is unclear if the sub-therapeutic concentrations (65 – 149 ng/L) of ciprofloxacin detected in the surface water (see Chapter 4, Section 4.3.3) select for ciprofloxacin resistant *E.coli*.

In this chapter the overall objective is to provide a better understanding of the effect ciprofloxacin discharged within treated wastewater effluent will have on the total *E.coli* population within receiving surface waters. In addition, the effect ciprofloxacin has on the prevalence of ciprofloxacin resistant *E.coli* will be investigated. Due to the complex variables within the environment that can influence the levels of resistance in surface waters, the use of microcosms was selected for this work. The relevance of *E.coli* as representative organisms to study the transfer of antibiotic resistance from wastewater to receiving surface waters and the importance of studying ciprofloxacin resistant profiles of *E.coli* in environmental waters has been discussed in Chapter 5, Section 5.1. The use of clinical breakpoints to interpret antibiotic susceptibility results from the environment may underestimate subtle changes in resistance (as discussed in Chapter 5, Section 5.4.2). Therefore, the *E.coli*/ciprofloxacin epidemiological cut off value (WT $\leq$ 64 μg/L) is used in this chapter. Epidemiological cut off values were introduced in Chapter 3, Section 3.4.1.

6.1.1.1 Small scale aquatic studies to monitor resistance in environmental waters

There are a number of laboratory aquatic studies to investigate the environmental fate and effects of medicinal products that have been recommended by The European Medicines Agency (2006) to assess and predict their potential risk to the environment (see Chapter 2, Section 2.5.4). However, within these guidelines there are no recommendations for more tailored studies to investigate the effects of antibiotics in aquatic ecosystems and the potential to exert a selective pressure for antibiotic resistant bacteria.

A number of researchers have carried out surveillance of the antibiotic resistance profiles of bacteria in surface waters to assess the impact of antibiotic contamination from sources

such as wastewater treatment plants (Moore et al., 2010; Servais et al., 2009; Leclercq et al., 2007; Goni-Urriza et al., 2000). Although this information is important for performing environmental risk assessments, collecting the data can be time consuming and the results can be influenced by environmental variability (e.g. climate and the catchment area characteristics). In addition, information on the association between antibiotic residues and the level of resistance cannot be obtained.

Laboratory microcosm studies use a compartment of the natural environment (soil or water) circumscribed under controlled conditions corresponding to natural ones (e.g. temperature, light and dissolved oxygen) for the assessment of the effects of a test substance on natural ecosystems (Barra Caracciolo et al., 2013). Laboratory microcosm studies and other small scale controllable systems (e.g. mesocosms) have been widely used as simple models of natural ecosystems to study the effects of pharmaceuticals and other organic pollutants (Rico et al., 2014; Shibata et al., 2014; Gonzalez-Pleiter et al., 2013; Boonstra et al., 2011; Pathak et al., 1994). The effects of antibiotics on the proliferation of antibiotic resistance in small scale aquatic systems have also been reported. Helt et al. (2012) monitored the prevalence of antibiotic resistant faecal bacteria in a constructed wetland system using qualitative phenotypic antibiotic susceptibility tests (disc diffusion). Whilst Munoz-Aguayo et al. (2007), assessed the proliferation of culturable antibiotic resistant bacteria in simulated river water systems exposed to chlortetracycline. In addition, some studies have set up systems to represent waters receiving agricultural waste such as swine and cattle waste and to monitor the presence of antibiotic resistant genes (Knapp et al., 2010; Zhang et al., 2009; Engemann et al., 2008).

6.1.1.2 Survival of E.coli in environmental waters

Previous studies have demonstrated that antibiotic-resistant *E.coli* may be found in different aquatic ecosystems including rivers (Sidrach-Cardona et al., 2014; Watkinson et al., 2007), streams (Akiyama et al., 2010), lakes (Jones et al., 1986) in addition to wastewaters (Silva et al., 2007). However, *E.coli* is a commensal bacterium from the gastrointestinal tracts of humans and vertebrate animals and the survival in secondary habitats such as river waters requires the ability to overcome environmental stresses. These include nutrient deprivation (Barcina et al., 1997), low temperature (Flint, 1987), salinity (Sinton et al., 2002), exposure to solar radiation (Sinton et al., 2002; McCambridge et al., 1981), competition with autochthonous microbial communities, and protozoan grazing (van Elsas et al., 2011; Barcina et al., 1997).

In addition, it has been assumed that antibiotic resistance confers a metabolic burden for bacteria. Therefore, antibiotic resistant bacteria will be out-competed by their sensitive (wild type) counterparts in the absence of selective pressures (Martinez, 2009). However, the fate of antibiotic resistant bacteria in the environment varies with the bacterial strain, the resistance elements (e.g. plasmid) and the habitat. Studies have demonstrated that the acquisition of resistance elements do not always affect the survival of the microorganism. For example, Enne et al. (2005) found that the fitness impact imposed on *E.coli* strain 345-2 RifC through the acquisition of antibiotic resistance elements was generally low or non-existent and indicated that once resistance was established it may be difficult to eliminate through reduction in antibiotic pressure. Flint (1987) demonstrated that the acquisition of

resistance plasmids R144-3 and R1*drd*-19 was not factor in the survival of *E.coli* in river water.

6.1.1.3 Ciprofloxacin resistance in natural waters

Fluoroquinolone resistance in *E.coli* has been discussed in Chapter 3, Section 3.3.5.2. Plasmid mediated ciprofloxacin resistance can occur through the acquisition of Qnr genes and the resulting minimum inhibitory concentrations values (MIC values) can increase between 8 and 64 fold following acquisition. Although the resulting MIC values may be lower than clinical breakpoint values, plasmids can facilitate higher quinolone resistance through the presence of more than one *Qnr* resistance gene (Robicsek et al., 2006). Their occurrence has been described in municipal biosolids and raw sewage (Kaplan et al, 2013) and in water borne *Aeromonas, Acinetobacter* and *Enterobacteria* species isolated from environmental waters (Marti et al., 2013; Figueira et al., 2011; Cattoir et al, 2008; Picao et al, 2008). Their prevalence in environmental waters indicates that the dissemination of plasmid mediated quinolone resistant genes do occur in environmental waters. The mechanisms of antibiotic resistance and the dissemination of antibiotic resistance are introduced in Sections 3.3.2 and 3.3.3, respectively of Chapter 3.

6.2 Material and methods

6.2.1 Method overview

In the present study, microcosms were constructed to simulate surface river water receiving wastewater treated effluent in order to monitor the ciprofloxacin resistance profiles of *E.coli* over 14 days. The microcosms were prepared from final treated wastewater effluent diluted

with surface river water (1:5 ratio) to simulate the dilution of wastewater effluent typically observed in receiving surface waters. In Europe, surface waters that receive wastewater treated effluent can have a dilution ratio within the range 1:1 to 1:5 (O'Brien et al., 2004). In addition, the microcosms were seeded with settled sewage to ensure the concentration of *E.coli* within the constructed microcosms were sufficient for the enumeration of total culturable *E.coli* and the associated ciprofloxacin resistant sub populations over the course of the experiment (14 days). The construction of the microcosms to assess the proliferation of ciprofloxacin resistant *E.coli* is described in Section 6.2.9.

Preliminary experiments were carried out to assess the effect of protozoa inhibitor compounds (cycloheximide and colchicine) on the survival of *E.coli* in surface water microcosms (see Section 6.2.8). The concentration of *E.coli* will decrease in surface waters due to predation by protozoa (McCambridge et al., 1981) and therefore the use of protozoa inhibitor compounds was important to ensure that the *E.coli* concentrations remained high enough for enumeration.

Assessing the impact of antibiotics on the proliferation of antibiotic resistant bacteria in surface waters is challenging as the natural background of resistance in surface waters (the proportion of resistance in environmental bacteria without anthropogenic impact), is unknown. Therefore in this study, environmentally relevant ciprofloxacin concentrations (already present and in the surface water/wastewaters collected for the microcosm studies and quantified by liquid chromatography-mass spectrometry) were investigated and compared to microcosms which were exposed to additional levels of ciprofloxacin (5, 10, 50 and 100 µg/L). The selected exposure concentrations (although sub-therapeutic) are greater

than those typically measured in surface waters receiving wastewater treated effluent discharges (see Chapter 4, Section 4.1.1) despite concentrations as high as 9.6 μg/L having been detected in surface waters by Feitosa-Felizzola et al. (2009). In addition, the selected exposure concentrations span the epidemiological cut off value (wild type without acquired ciprofloxacin resistance $\leq$ 64 μg/L) established by the European Committee of Antimicrobial Susceptibility Testing (EUCAST, 2012) which is used to distinguish between *E.coli* with and without acquired ciprofloxacin resistance. The working hypothesis was that within microcosms exposed to ciprofloxacin levels greater than the epidemiological cut off value, a more pronounced effect on the proportion of *E.coli* with acquired resistance would be observed.

Frequently in studies investigating the antibiotic resistance profiles of bacteria in environmental waters, the target bacteria are processed for isolation, identification and resistance to antibiotics (Ferreira da Silva et al., 2007; Goni-Urriza et al., 2000). This can be a time consuming and costly process when used for broad spatial or temporal assessments of antibiotic resistance patterns in environmental waters. Novo et al. (2013) and Watkinson et al. (2008) both reported that using the membrane filtration method incorporating a chromogenic media specific for the detection of *E.coli* supplemented with antibiotics can be reliably used for the enumeration of antibiotic resistant populations of *E.coli* in environmental waters. This method was employed for this work using tryptone bile x glucuronide (TBX) supplemented to final nominal ciprofloxacin concentrations of 16, 32, 64, 125 and 2000 μg/L. TBX media was selected for the work in this chapter as it has been reported that it is a highly specific medium for *E.coli* (Hansen et al., 1984). The antibiotic concentrations were selected to determine the level of resistance to various concentrations

of ciprofloxacin (both above and below inhibitory concentrations) and to estimate the ciprofloxacin minimum inhibitory concentrations among the total culturable *E.coli* population. In addition, the concentrations of ciprofloxacin were chosen to determine the proportion of total culturable *E.coli* with acquired ciprofloxacin resistance (according to the epidemiological cut off value) and to identify if any *E.coli* strains exhibited minimum inhibitory concentration values greater than the ciprofloxacin clinical breakpoint value (resistant > 1000 μg/L).

Typically, culture techniques including the agar dilution method used to determine antibiotic minimum inhibitory concentration values are carried out using a reference media (e.g. Mueller Hinton) (Andrews 2001). The techniques available to determine bacterial resistance to antibiotics were introduced in Chapter 3, Section 3.4. Medium supplements used to support the growth of specific bacteria or to inhibit the growth of non-target bacteria may influence the minimum inhibitory concentration value determined. Therefore, the use of TBX supplemented with ciprofloxacin for the determination of ciprofloxacin resistant levels of *E.coli* was evaluated. A total of fifty *E.coli* strains taken from wastewater and surface water samples with known ciprofloxacin minimum inhibitory concentration values (achieved using antibiotic gradient strips as described in Chapter 5) were re-tested using TBX supplemented with different concentrations of ciprofloxacin (see Section 6.2.6).

TBX has been reported as a highly specific chromogenic medium for the detection of *E.coli*. The media employs a chromogenic substrate which detects glucuronide activity which has been reported to be 94 – 96 % of *E.coli* (Manafi, 2000). However, glucuronide activity may be present in other bacterial species (see Chapter 5, Section 5.3.2) including *Citrobacter*

Freundii which are present in surface waters (Alonso et al. 1996). In addition, supplementation with ciprofloxacin may affect the performance of TBX to detect *E.coli* within surface waters whilst inhibiting non-target species. Therefore, a trial was conducted using surface water samples to assess the performance of TBX to selectively detect *E.coli* resistance to different levels of ciprofloxacin. In addition, the levels of ciprofloxacin resistance determined using this method were verified.

6.2.2 Media and reagents

Tryptone bile X-glucuronide (TBX) media (CM0945) and Mueller Hinton (MH) agar (CM0337) were purchased from Oxoid Ltd. Ciprofloxacin (≥ 98 % HPLC) was purchased from Sigma-Aldrich Company Ltd, UK. Colchicine (97%), sodium hydroxide (98 % CertiFied AR) and cycloheximide (95%) were purchased from Fisher Scientific UK Ltd and ciprofloxacin Etest® strips (0.002-32 µg/mL) were purchased from Biomerieux Ltd, UK.

6.2.2.1 Preparation of ciprofloxacin stock solutions

A 10 g/L stock solution of ciprofloxacin was prepared by dissolving 10g in sterile deionised water. Whilst the solution was stirred, a 0.1 M sodium hydroxide solution (prepared with sterile water) was added drop by drop until the antibiotic dissolved. Sterile water was then used to make up to 1 L, inverted to fully mix and filtered (Millipore, Millex sterile, syringe filter 0.2 µm). The stock solution was stored in 100 mL aliquots at -30 °C until required. Working stock solutions for microcosm or media preparation were prepared by diluting the 10 g/L stock with sterile deionised water on the day of use.

6.2.2.2 Preparation of TBX media supplemented with ciprofloxacin

Batches of Tryptone bile X-glucuronide (TBX) media were prepared by dissolving 36.6 g of TBX in 1 L sterile water followed by autoclaving at 121 °C. Different concentrations of ciprofloxacin in the molten agar were prepared according to the dilution details given in Table 6-1.

Table 6-1: Preparation of ciprofloxacin solutions used to prepare TBX supplemented with 16, 32, 64, 125 and 2000 µg/L ciprofloxacin.

Ciprofloxacin stock used (mg/L)	Volume of stock (mL)	Diluted with sterile water (mL)	Ciprofloxacin concentration obtained (mg/L)	Final nominal concentration in TBX after addition to 950 mL of agar (µg/L)
10,000	0.1	9.9	100	-
100	1.0	9.0	10	-
10	1.6	48.4	0.32	16
10	3.2	46.8	0.64	32
10	6.4	43.6	1.28	64
10	12.5	37.5	2.50	125
100	20	30	40	2000

The molten agar supplemented with ciprofloxacin (8 mL) was then dispensed into 50 mm petri dishes for membrane filtration testing (see Section 6.2.5). In addition, 50 mL of each ciprofloxacin concentration was dispensed into 120 mm square petri dishes for the evaluation of tryptone bile X-glucuronide media for *E.coli* ciprofloxacin susceptibility testing.

6.2.3 Microcosm preparation

Microcosms were established in 4L conical flasks to simulate surface water receiving treated wastewater effluent. Flasks were closed with a silicone stopper (Bugstopper™, Whatman, UK) to prevent the evaporation of water and to prevent organisms entering the flasks whilst allowing a free passage of air to vent the flasks. The flasks were gently agitated using magnetic stirrers to facilitate oxygen transfer to the water from the flask headspace, so that aerobic conditions were adequately maintained. To prevent UV- light inactivation of *E.coli*, the microcosms were wrapped in aluminium foil. The microcosms were located in a laboratory with a controlled temperature of 21 °C.

The surface water and wastewaters used in the microcosms were collected on the same day that the microcosms were to be established. 50 L of surface water was collected up-stream of the final treated effluent discharge point of a large urban wastewater plant. 5 L of settled sewage and 10 L of final treated effluent were collected from sampling points prior to and after the activated sludge treatment system (described in Chapter 4 Section 4.2.2). The surface water, effluent and settled sewage were collected in 10 L jerricans (Fisher scientific, UK) previously sterilised by irradiation and stored in ice during return to the laboratory. The surface water (37.5 L), final treated effluent (7.5 L) and settled sewage (2.25 L) were mixed thoroughly in sterile containers before distributed to each of the 4 L conical flasks.

More details on the construction of the surface water microcosms to assess the use of protozoa inhibitor compounds on the survival of *E.coli* are given in Section 6.2.8. The microcosm experiment set up to monitor the proliferation of ciprofloxacin resistant *E.coli* is described in Section 6.2.9.

6.2.4 Enumeration of total culturable *E.coli*

The concentrations of total culturable *E.coli* present within each microcosm were enumerated using the membrane filtration technique (previously described in Chapter 5, Section 5.2.4.1). However, tryptone bile x-glucuronide (TBX) media was employed as it is specific for the detection of *E.coli.* Water samples from each microcosm were serially diluted (ten-fold) and 100 mL aliquots were filtered through 0.45 µm, 47 mm, cellulose nitrate membrane filters. The membrane filters were then placed on to TBX (in 50 mm petri dishes) and incubated for 4 h at 30°C, followed by 18 - 24 h at 44°C. *E.coli* produce blue/green colonies on TBX and therefore all blue/green colonies were enumerated.

6.2.5 *Enumeration of E.coli* resistant to 32, 64, 125 and 2000 µg/L ciprofloxacin

The counts of *E.coli* resistant to different concentrations of ciprofloxacin were enumerated by the membrane filtration technique (previously described in Chapter 5, Section 5.2.4.1) using TBX media supplemented with ciprofloxacin (to final nominal concentrations 16, 32, 64, 125, and 2000 µg/L). The ciprofloxacin concentrations were selected as they span the concentrations that define sensitive and wild type strains up to concentrations greater than those used to define resistant and non-wild type strains. The *E.coli*/ciprofloxacin clinical breakpoint and the epidemiological cut off values defined by the European Committee of Antimicrobial Susceptibility Testing and used for interpretation in this study are given in Chapter 5, Section 5.2.7.1.

From the diluted samples used to enumerate the total culturable *E.coli* (described in Section 6.2.4), five additional 100 mL aliquots were filtered through 0.45 µm, 47 mm, cellulose nitrate membrane filters and placed on to TBX with the final nominal ciprofloxacin

concentrations of 16, 32, 64, 125 and 2000 µg/L). Following incubation for 4 h at 30°C, followed by 18 - 24 h at 44°C, blue/green colonies on each filter were enumerated.

6.2.5.1 Interpretation of E.coli counts enumerated on TBX supplemented with ciprofloxacin

The proportion (%) of total culturable *E.coli* resistant to 16, 32, 64, 125 and 2000 µg/L ciprofloxacin within each microcosm was estimated by comparing the *E.coli* counts (CFU/100 mL) on TBX supplemented with the appropriate level of ciprofloxacin with the corresponding counts of total culturable *E.coli* on TBX without ciprofloxacin using Equation 6-1.

Equation 6-1

$$\%\ \text{resistance to x µg/L ciprofloxacin} = \frac{[E.coli]\ \text{on TBX supplemented with x µg/L ciprofloxacin}}{[E.coli]\ \text{on TBX}}$$

Where:

[*E.coli*] = concentration of *E.coli* (CFU/100 mL)

x = final nominal ciprofloxacin concentration of TBX media

The concentration (CFU/100 mL) of *E.coli* enumerated on each TBX plate supplemented with ciprofloxacin and the corresponding proportion (%) of the total culturable *E.coli* population were interpreted as follows:

MIC_{50}: The TBX ciprofloxacin concentration that inhibits ~ 50 % of the total culturable *E.coli* population within each microcosm

MIC_{90}: The TBX ciprofloxacin concentration that inhibits ~ 90 % of the total culturable *E.coli* population within each microcosm

% acquired resistance: The proportion (%) of total culturable *E.coli* determined on TBX supplemented with a final nominal concentration of 125 μg/L ciprofloxacin. According to the epidemiological cut off value, *E.coli* strains with ciprofloxacin minimum inhibitory concentration value > 64 μg/L are non-wild type with acquired resistance. Therefore TBX supplemented with 125 μg/L ciprofloxacin was selected to determine the proportion of the total *E.coli* population with acquired ciprofloxacin resistance.

6.2.6 Evaluation of TBX supplemented with ciprofloxacin to determine ciprofloxacin MIC values.

To evaluate TBX supplemented with ciprofloxacin for the determination of *E.coli* ciprofloxacin minimum inhibitory concentration (MIC) values, a selection of *E.coli* strains with known ciprofloxacin MICs (determined using ETEST®) were selected. The ciprofloxacin MICs determined using TBX were then compared to those obtained by the ETEST method®

To obtain ciprofloxacin MIC values using TBX, the agar dilution method reported by EUCAST (2000) was used. A suspension (comparable to a 0.5 McFarland turbidity standard) of each isolate (fresh culture) was prepared in a 0.85 % sodium chloride solution and applied onto TBX media supplemented with twofold concentrations of ciprofloxacin (within the range 16 – 2000 μg/L). TBX without ciprofloxacin was used as a positive control for *E.coli* growth. The media was incubated for 4 h at 30°C, followed by 18 - 24 h at 44°C. Following incubation, the MIC value was determined from TBX with a final ciprofloxacin concentration that visibly inhibited the growth of the isolate.

The MIC values determined by both methods were analysed using the Mann Whitney test (data did not have a normal distribution with or without data transformation). In addition, the MIC values determined using both methods were analysed for essential agreement. Essential agreement describes the proportion (%) of MIC values obtained for the fifty isolates by the two antibiotic susceptibility testing methods that are within ± one two-fold concentration (Canton et al., 2000).

6.2.7 Application of TBX supplemented with ciprofloxacin to detect *E.coli* within surface waters

The membrane filtration technique employing TBX supplemented with ciprofloxacin was trialled to detect *E.coli* resistant to different concentrations of ciprofloxacin within surface water. Surface water samples were collected (in duplicate) down-stream relative to a treated effluent discharge point of a large urban wastewater treatment plant (the description and map of the sampling site are given in Chapter 5, Section 5.2.2). *E.coli* with and without acquired ciprofloxacin resistance were previously detected at this sampling location (see Chapter 5, Section 5.3.4.3) and therefore the sampling site was selected for this trial.

A selection of blue/green colonies (n = 131) were taken from membrane filters incubated on TBX supplemented with final nominal concentrations of ciprofloxacin (16, 32, 64, 125 and 2000 µg/L). The identity of each colony was confirmed using MALDI-TOF-MS analysis (according to the method given in Chapter 5, Section 5.2.6.2). In addition, the identity of a selection of blue/green colonies (n = 30) from membrane filters incubated on TBX without ciprofloxacin were verified using MADI-TOF-MS analysis.

All 131 colonies taken from TBX supplemented with ciprofloxacin to a final ciprofloxacin concentration of *x* μg/L were presumed to produce ciprofloxacin minimum inhibitory concentration (MIC) values > *x* μg/L. To verify the level of ciprofloxacin resistance determined using TBX supplemented with ciprofloxacin, the ciprofloxacin MIC values for each isolate were determined using ETEST® (according to the method given in Chapter 5, Section 5.2.7). If the ciprofloxacin MIC value exceeded the TBX supplemented concentration the isolate was taken from, the level of ciprofloxacin resistance was considered as verified.

6.2.8 Evaluation of protozoa inhibitor compounds on the survival of *E.coli* in microcosms

The effect of different protozoa inhibitor treatments on the survival of *E.coli* in surface water microcosms was assessed using twelve flasks set up as described in Section 6.2.3. Three different protozoa inhibitor treatments and a control (no protozoa inhibitors used) were assessed in triplicate. The protozoa inhibitors used, the quantity used and when the inhibitors were administered to the microcosms are described in Table 6-2. Water samples for the enumeration of *E.coli* (using the method outlined in Section 6.2.4) were taken on the day the microcosms were set up (day 0), and then on days 3, 7, 10 and 14. The enumeration of *E.coli* within each microcosm was performed in triplicate.

Table 6-2: Protozoa inhibitors used to increase the survival of *E.coli* in surface water microcosms.

Protozoa inhibitor	Quantity added to microcosm (mg)	Final volume of microcosm (L)	Final concentration of inhibitor in microcosm (mg/L)	Administered (day)
Control	-	2	-	-
Cycloheximide	1000	2	500	[a] 0
Periodic addition of cycloheximide	1000 per addition	2	1000	[a] 0 and 3, 7 and 10
Cycloheximide and colchicine	400 (cycloheximide) 200 (colchicine)	2	200 (cycloheximide) 100 (colchicine)	[a] 0

-No protozoa added. [a] Protozoa inhibitor added at the commencement of the microcosms

6.2.9 Microcosm experiment to assess the proliferation of ciprofloxacin resistant *E.coli*

For this experiment, fifteen flasks each containing 3L of a surface water/wastewater mix were set up as described in Section 6.2.3. To inhibit protozoa, colchicine (300 mg) and cycloheximide (600 mg) were added to each flask to give final concentrations of 200 and 100 mg/L. The decision to use colchicine and cycloheximide to inhibit protozoa was based on the results presented in Section 6.3.1. Five groups of triplicate microcosms (see Table 6-3) were used to investigate the ciprofloxacin resistance profiles of *E.coli* exposed to five different levels of additional ciprofloxacin (0, 5, 10, 50 and 100 μg/L). Appropriate volumes of a ciprofloxacin stock solution were added to the microcosms to reach nominal ciprofloxacin exposure concentrations as outlined in Table 6-3. The exposure concentration defines the concentration added to the microcosm which is additional to the concentration already present in the surface water/wastewater sample within each microcosm. The concentration already present in the surface water/ wastewater sample was determined by liquid chromatography mass spectrometry as outlined in Section 6.2.9.1.

Table 6-3: Administration of ciprofloxacin to constructed microcosms.

[a] Exposure level of additional ciprofloxacin (μg/L)	Spiking volume of 100 mg/L ciprofloxacin stock solution (mL)	Volume of homogenised sample in flask (L)
0	0	3
5	0.15	3
10	0.3	3
50	1.5	3
100	3.0	3

[a] nominal concentration that is additional to the concentration already present in the sample.

The microcosm experiment was initially carried out in November 2012 and repeated in July 2013. Samples from each microcosm for bacteriological analysis (enumeration of total culturable *E.coli* and *E.coli* resistant to ciprofloxacin) were taken on day 0 (the day the microcosms were established, before additional ciprofloxacin exposure). Additional samples were taken on days 1, 7, 10 and 14 (post ciprofloxacin exposure). During the experiment carried out in July 2013, additional samples for bacteriological analysis were also taken on day 3 (post additional ciprofloxacin exposure).

6.2.9.1 Chemical analysis

Aliquots of the surface water/wastewater mixed sample used for the microcosms were taken for quantification by liquid chromatography mass spectrometry (see the method description in Chapter 4, Section 4.2.5) to determine the ciprofloxacin levels already present in the microcosm surface water/wastewater mix. Water quality parameters (pH, total suspended solids (TSS) and dissolved oxygen (DO)) were measured for each microcosm at the commencement of the experiment. Dissolved oxygen (DO) was subsequently measured at daily intervals to ensure aerobic conditions were maintained (> 65 % saturation). TSS was determined by the method described in Chapter 4, Section 4.2.4.

Kruskal-Wallis one-way analysis of variance was used to ascertain if the water quality parameters differed significantly. This non-parametric test was used as the acquired data was not normally distributed.

6.2.9.2 Bacteriological analysis

Duplicate samples (60 mL) for the determination of *E.coli* ciprofloxacin resistance profiles were extracted using a sterile pipette and stored in 100 mL sterile polypropylene containers at 4 °C until analysis (within 4 h) on the day the experiment commenced (i.e. before exposure to additional ciprofloxacin) and then on days 1, 7, 10 and 14. In the second microcosm experiment carried out in July 2013, samples for bacterial analysis were also extracted on day 3.

6.2.9.3 Statistical analysis

Analysis of difference of the proportion of total culturable *E.coli* with acquired ciprofloxacin resistance among microcosms exposed to different levels of ciprofloxacin was carried out using one-way ANOVA with post hoc Tukey analysis. Additionally, the proportion of ciprofloxacin acquired resistance within the constructed microcosms over time was performed using one-way ANOVA with post hoc Tukey analysis.

6.3 Microcosm bacteriological analysis

6.3.1 The effect of protozoa inhibitors on the survival of *E.coli* in surface water microcosms

Protozoa can impair the survival of *E.coli* in natural waters through grazing (Enzinger et al., 1976). The effect of three different protozoa inhibitor treatments (cycloheximide, periodic addition of cycloheximide and a combination of cycloheximide and colchicine) on the survival of *E.coli* within surface water microcosms (mixed with wastewater) were assessed in triplicate over 14 days. In addition, triplicate surface water microcosms without the addition of a protozoa inhibitor compound were monitored as a control.

At the commencement of the experiment (day 0), the concentrations of *E.coli* were similar (one-way ANOVA - $p > 0.05$) in all microcosms (within the range 5.0 – 5.4 $\log_{10}$ CFU/100 mL) followed by a progressive decrease over 14 days (Figure 6-1). A larger decrease in the concentration of *E.coli* was observed in the control microcosms (no protozoa inhibitor compounds added) compared to the other microcosms. Thus indicating protozoa do impact the survival of *E.coli* in natural waters.

However, the addition of a both cycloheximide and colchicine into the surface water microcosms at day 0 resulted in significantly higher concentrations (average ± standard deviation = 3.6 ± 0.1 $\log_{10}$ CFU/100 mL) of *E.coli* on day 14 compared to all other microcosms (one-way ANOVA – $p < 0.05$). Therefore, cycloheximide and colchicine were used to inhibit protozoa in subsequent microcosm experiments.

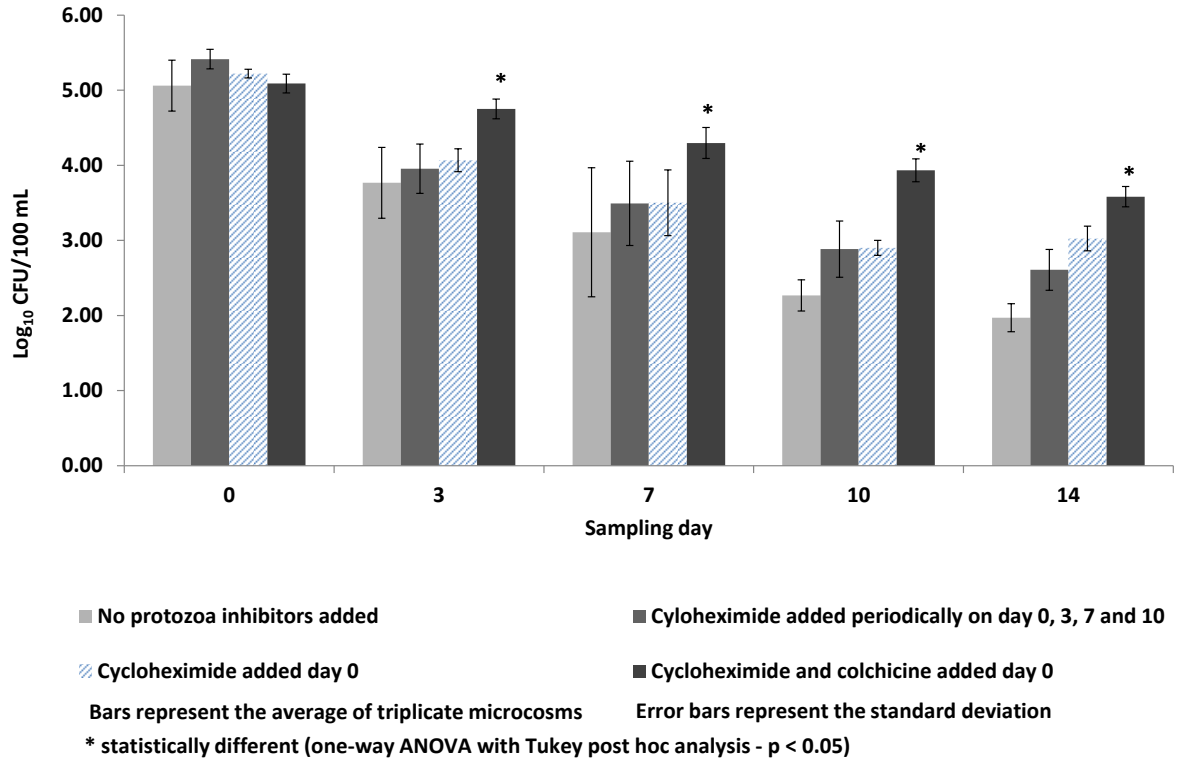


Figure 6-1: Changes in the total culturable *E.coli* concentrations within microcosms exposed to different protozoa inhibitor treatments

6.3.2 Evaluation of TBX supplemented with ciprofloxacin to determine ciprofloxacin minimum inhibitory concentration values of *E.coli*.

The ciprofloxacin minimum inhibitory concentration (MIC) values of fifty *E.coli* isolates were determined by two methods: the agar dilution method employing TBX supplemented with ciprofloxacin and with ETEST® antibiotic gradient strips. A summary of the MIC values obtained by both methods is given in Table 6-4.

Mann Whitney analysis of the determined MIC values did not find any significant difference between the two methods (Table 6-4). The proportion of MIC values determined using TBX that were in essential agreement with the ETEST® method (98.0 %) was high. Furthermore, the proportion of isolates that were identified as non-wild type (with acquired ciprofloxacin

resistance according to the epidemiological cut off value) by the two methods differed by only 2 %. Therefore the use of TBX for the determination of *E.coli* ciprofloxacin MIC values was considered appropriate for use in this study.

Table 6-4: Comparison of the ciprofloxacin MIC values determined for fifty *E.coli* isolates by TBX supplemented with ciprofloxacin and the ETEST®.

	TBX	ETEST®
MIC range (μg/L)	16 - 2000	16 - 2000
Median (μg/L)	64	64
% ≤ ECOff	54.0	56.0
% > ECOff	46.0	44.0
Identical MIC values (%)	80.0	
[a] % Essential agreement	98.0	
Mann Whitney	p = 0.876	

[a] Essential agreement – the proportion of MIC values by the two methods that are within ± one two fold concentration

6.3.3 Application of TBX supplemented with ciprofloxacin to detect *E.coli* resistant to different concentrations of ciprofloxacin.

Surface water samples were processed using the membrane filtration technique employing TBX supplemented with different concentrations of ciprofloxacin (0, 16, 32, 64, 125 and 2000 μg/L). A selection of blue/green colonies (presumptive *E.coli*) was isolated in order to verify their identity using MALDI-TOF-MS analysis. In addition, for each isolate taken from TBX supplemented with ciprofloxacin, the ciprofloxacin MIC values were determined. The proportion of isolates that confirmed as *E.coli* is given in Table 6-5. The proportion of

isolates with ciprofloxacin MIC values exceeding the TBX supplemented concentration is also presented in Table 6-5.

Table 6-5: The proportion (%) of isolates taken from surface water using TBX, verified as *E.coli* and the proportion of isolates that produced ciprofloxacin MIC values exceeding the TBX supplemented concentration.

[a] TBX ciprofloxacin concentration (µg/L)	Number of isolates tested	% of total presumptive *E.coli* [b] identity verified as *E.coli*	Ciprofloxacin MIC value > TBX ciprofloxacin concentration
0	30	96.7	ND
16	20	100.0	100.0
32	40	90.0	100.0
64	20	100.0	100.0
125	31	93.5	100.0
2000	20	100.0	100.0

[a] TBX media was supplemented with the stated final nominal concentrations. [b] Isolates not verified as *E.coli* were identified as *C. freundii*. ND – not done

A high proportion (> 90.0 %) of presumptive *E.coli* isolates taken from TBX supplemented with different concentrations of ciprofloxacin was verified as *E.coli*. In addition, all isolates taken from TBX supplemented with ciprofloxacin produced ciprofloxacin MIC values exceeding the TBX supplemented concentration. The isolates that were not verified as *E.coli* were identified as *Citrobacter Freundii*. Similar false positive identifications using chromogenic media specifically for the detection of *E.coli* and coliforms have been reported by Alonso et al. (1996).

The results show that TBX supplemented with ciprofloxacin is highly specific for the detection of *E.coli* within surface water and is suitable for the detection of *E.coli* resistant to different levels of ciprofloxacin.

6.3.4 Microcosm experiments to assess the proliferation of ciprofloxacin resistant *E.coli*

Microcosm experiments to assess the proliferation of ciprofloxacin resistant *E.coli* in surface waters were carried out in November 2012 and repeated in July 2013. The counts of total culturable *E.coli* and of *E.coli* resistant to different levels of ciprofloxacin were enumerated within all microcosms over the course of 14 days. Appropriate physicochemical parameters were also monitored during each microcosm experiment.

6.3.4.1 Microcosm physicochemical parameters

Table 6-6 summarises the water quality parameters observed in the microcosm experiments conducted in November 2012 and in July 2013. The dissolved oxygen levels were maintained throughout the duration of the experiments (7.9 – 8.1 mg/L) and ensured the preservation of aerobic conditions (Kruskal-Wallis; p = > 0.05) amongst all microcosms. The pH values measured in the all of the constructed microcosms were comparable (7.4 – 7.6) (Kruskal-Wallis; p = > 0.05) and within the range (≥ 6.0 – ≤ 9.0) expected in rivers in the UK for the support of biota (UK Technical Advisory Group on the Water Framework Directive (UKTAG), 2008). The total suspended solid (TSS) concentrations were consistent for the different series of experiments (Kruskal-Wallis; p = > 0.05) performed in November 2012 and July 2013. However elevated TSS concentrations (150.0 – 155.0 mg/L) were observed for the July experiment compared to those observed in November 2012 (90.6 – 96.3 mg/L).

Table 6-6: Water quality parameters measured within microcosms exposed to different additional concentrations of ciprofloxacin (0, 5, 10, 50 and 100 µg/L), in the experiments carried out in November 2012 and July 2013

Exposure level of additional ciprofloxacin (µg/L)	Water quality parameters (mean ±standard deviation)			
	TSS (mg/)	pH	DO (mg/L)	[a] Ciprofloxacin (ng/L)
November 2012 experiment				
0	96.3 ± 3.1	7.4 ±0.1	8.1 ± 0.1	257 ± 16
5	93.3 ± 2.5	7.5 ± 0.1	8.0 ± 0.1	
10	92.0 ± 1.1	7.5 ± 0.2	8.0 ± 0.1	
50	95.2 ± 0.6	7.5 ± 0.1	8.0 ± 0.1	
100	90.6 ± 2.1	7.4 ± 0.1	7.9 ± 0.1	
July 2013 experiment				
0	154.0 ±5.6	7.6 ± 0.0	7.9 ± 0.1	161 ± 81
5	155.0 ±4.7	7.6 ± 0.1	7.9 ± 0.1	
10	150.0 ± 2.7	7.6 ± 0.0	7.9 ± 0.1	
50	154.0 ± 2.0	7.6 ± 0.1	7.9 ± 0.1	
100	152.1 ± 2.9	7.5 ± 0.0	7.9 ± 0.1	

[a] Levels of ciprofloxacin detected in the mixed surface water/sewage sample prior to exposure to additional levels of ciprofloxacin. TSS represents the mean total suspended solids concentration of triplicate microcosms measured on day 0 when the experiment was commenced. pH is the mean value of triplicate microcosms measured on day 0. DO represents the mean (n = 42) dissolved oxygen level measured over the course of the experiment (days 0, 1, 7, 10 and 14).

The concentrations of ciprofloxacin measured in the surface water/wastewater mixed samples used for the microcosm experiments were 257 ± 16 ng/L (November 2012) and 161 ± 81 ng/L (July 2013). These values are comparable to those previously detected in surface waters down-stream from the WWTP treated effluent discharge point (Chapter 4, Section

4.3.3) and are comparable to concentrations reported in surface waters in other studies (Tuc Dinh et al., 2011 and Watkinson et al., 2009).

6.3.4.2 Enumeration of total culturable E.coli in surface water microcosms exposed to different levels of ciprofloxacin

The concentrations of total culturable *E.coli* enumerated within all microcosms over the course of the 14 day experiments carried out in November 2012 and in July 2013 are shown in Figure 6-2 A and B. For both experiments, total culturable *E.coli* were present in similar concentrations for all microcosms on the first sampling date (Day 0 – before exposure to additional ciprofloxacin). However, the initial concentration of total culturable *E.coli* were slightly higher (in the order of 5.9 $\log_{10}$ CFU/100 mL) among microcosms analysed in July 2013 compared to November 2012 (in the order of 4.8 $\log_{10}$ CFU/100 mL). This could, in part, be explained by the higher concentration of suspended solids that were present in microcosms analysed in July 2013 (see Table 6-6).

The counts of total culturable *E.coli* decreased over the course of both experiments. For the experiment carried out in July 2013, the concentration decreased to non-detectable limits in microcosms exposed to 100 μg/L additional ciprofloxacin on day 10. Additionally, total culturable *E.coli* could not be detected within microcosms exposed to 5 and 50 μg/L ciprofloxacin by day 14. During the experiment in November 2012, total culturable *E.coli* could not be enumerated in microcosms exposed to 100 μg/L additional ciprofloxacin on day 14.

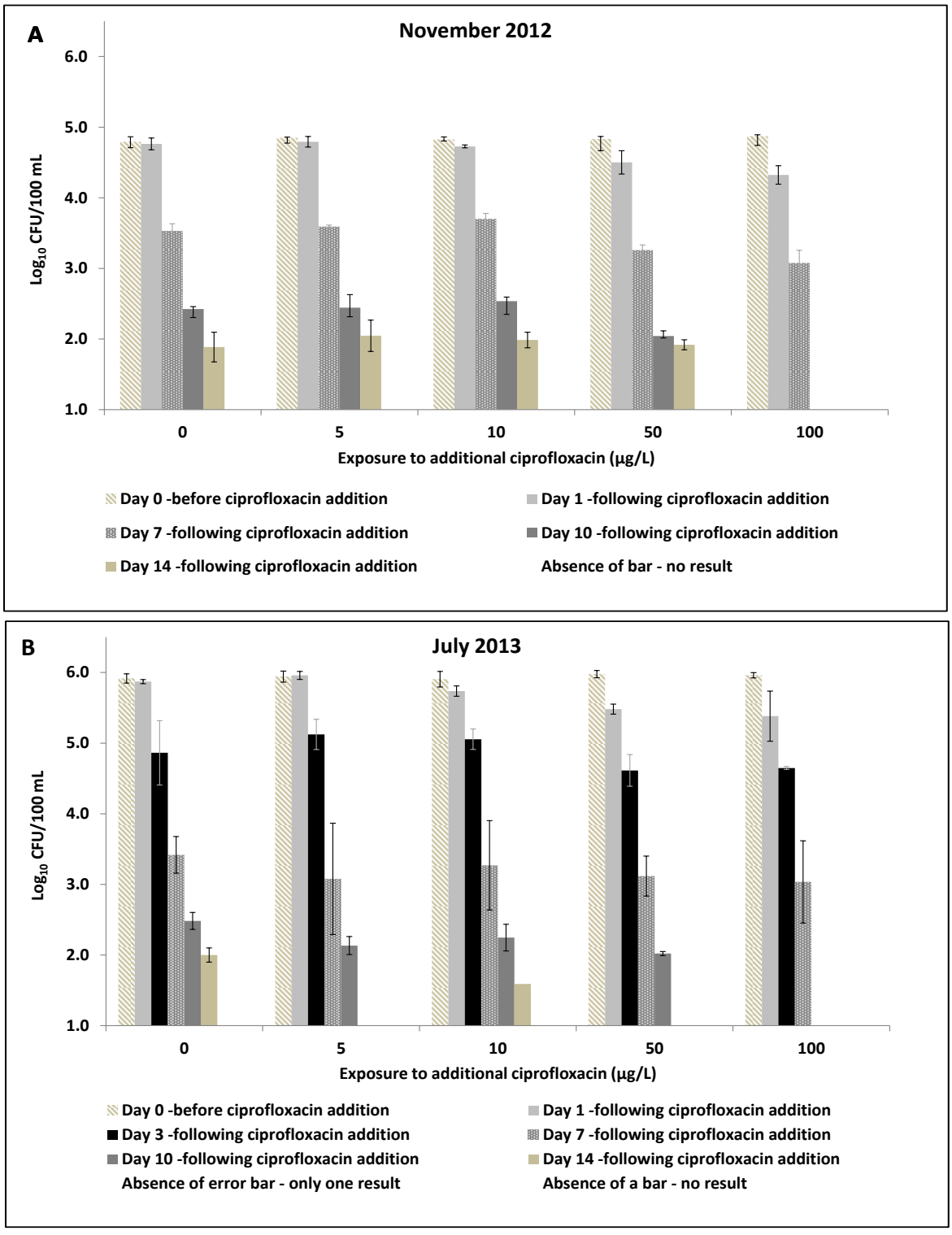


Figure 6-2: The enumeration of total culturable *E.coli* (average ± standard deviation) within the constructed microcosms exposed to different levels of additional ciprofloxacin on five sampling dates in November 2012 (A) and six sampling dates in July 2013 (B).

A closer look at the concentrations of total culturable *E.coli* for both experiments (November 2012 and July 2013) revealed that the reduction (%) in concentration between sampling day 0 (before additional ciprofloxacin exposure) and sampling day 1 (after ciprofloxacin exposure) was significantly greater ($p < 0.05$) within microcosms exposed to 50 and 100 μg/L ciprofloxacin compared to the other microcosms (as shown in Table 6-7). Thus demonstrating an impact of exposure to additional ciprofloxacin at concentrations greater than 50 μg/L on the total culturable *E.coli* population. The percentage reduction of *E.coli* was calculated using Equation 6-2.

Table 6-7: Reduction of *E.coli* (%) in constructed microcosms after 1 day following exposure to either, 0, 5, 10, 50 or 100 μg/L additional ciprofloxacin.

Exposure level of additional ciprofloxacin (μg/l)	[a] Mean % reduction ± standard deviation	
	November 2012	July 2013
0	6.5 ± 9.4	1.4 ± 5.6
5	11.3 ± 14.6	5.7 ± 12.4
10	20.1 ± 6.5	32.0 ± 6.2
50	*51.3 ± 17.2	*65.8 ± 3.9
100	*70.9 ± 9.9	*70.0 ± 20.2

[a] Calculated from Equation 6-2.* Statistical difference ($p < 0.05$) between microcosm groups (One-way ANOVA with Tukey post hoc analysis).

Equation 6-2:

$$\text{Reduction following ciprofloxacin exposure (\%)} = \frac{\text{day 0 - day 1}}{\text{day 0}} \times 100$$

Where:

day 0 = total culturable *E.coli* (CFU/100 mL) measured on day 0 (before exposure to ciprofloxacin)

day 1 = total culturable *E.coli* (CFU/100 mL) measured on day 1 (after exposure to ciprofloxacin).

During the experiment carried out in November 2012, the reduction in total culturable *E.coli* between the subsequent sampling days was similar (within the range 92.0 – 99.2 %) among all microcosms (data not shown). Similarly, the reduction in total culturable *E.coli* between subsequent sampling days (78.3 – 98.4 %) was similar among microcosms monitored in July 2013.

6.3.4.3 Minimum inhibitory concentration determination

The ciprofloxacin MIC_{50} and MIC_{90} values determined for the total *E.coli* population within all microcosms over the course of the experiments commenced in November 2012 and in July 2013 are summarised in Tables 6-8 and 6-9. In addition, the range of TBX supplemented ciprofloxacin concentrations for which *E.coli* could be detected is given. Not determined (ND) is recorded where *E.coli* counts on TBX supplemented with ciprofloxacin were below detectable limits.

Table 6-8: Ciprofloxacin minimum inhibitory concentration (MIC) values for the *E.coli* population within microcosms before (day 0) and after (days 1, 7, 10 and 14) exposure to different levels of additional ciprofloxacin during the experiment commenced November 2012.

Exposure level of additional ciprofloxacin (μg/L)	Sample day	Range (μg/L)	MIC_{50} (μg/L)	MIC_{90} (μg/L)
0 μg/L	0	16 - 2000	16	125
	1	16 - 2000	16	125
	7	16 -2000	16	125
	10	16 - 64	16	ND
	14	ND	ND	ND
5 μg/L	0	16 - 2000	16	125
	1	16 - 2000	16	125
	7	16 - 2000	16	2000
	10	16 - 64	16	ND
	14	ND	ND	ND
10 μg/L	0	16 - 2000	16	125
	1	16 - 2000	16	2000
	7	16 - 2000	16	2000
	10	16 - 64	16	ND
	14	ND	ND	ND
50 μg/L	0	16 - 2000	16	125
	1	16 - 2000	125	>2000
	7	16 - 2000	125	>2000
	10	16 - 64	ND	ND
	14	ND	ND	ND
100 μg/L	0	16 - 2000	16	125
	1	16 - 2000	125	>2000
	7	16 - 2000	125	>2000
	10	ND	ND	ND
	14	ND	ND	ND

MIC^{50} is an estimate of the concentration of antibiotic that inhibits 50% of the population of total *E.coli*. MIC^{90} is an estimate of the concentration of antibiotic that inhibits 90% of the population of total *E.coli*. ND – could not be determined from the counts on TBX supplemented with ciprofloxacin.

Table 6-9: Ciprofloxacin minimum inhibitory concentration (MIC) values for the *E.coli* population within microcosms before (day 0) and after (days 1, 3, 7, 10 and 14) exposure to different levels of additional ciprofloxacin during the experiment commenced in July 2013.

Exposure level of additional ciprofloxacin (µg/L)	Sample day	Range (µg/L)	MIC_{50} (µg/L)	MIC_{90} (µg/L)
0 µg/L	0	16 - 2000	16	125
	1	16 - 2000	16	125
	3	16 - 2000	16	125
	7	16 - 2000	16	125
	10	16 - 125	16	ND
	14	ND	ND	ND
5 µg/L	0	16 - 2000	16	125
	1	16 - 2000	16	125
	3	16 - 2000	16	2000
	7	16 - 2000	16	2000
	10	16 - 125	16	ND
	14	ND	ND	ND
10 µg/L	0	16 - 2000	16	125
	1	16 - 2000	16	2000
	3	16 - 2000	16	2000
	7	16 - 2000	16	2000
	10	ND	ND	ND
	14	ND	ND	ND
50 µg/L	0	16 - 2000	16	125
	1	16 - 2000	64	>2000
	3	16 - 2000	125	>2000
	7	16 - 2000	125	>2000
	10	16 - 2000	125	ND
	14	ND	ND	ND
100 µg/L	0	16 - 2000	16	125
	1	16 - 2000	125	>2000
	3	16 - 2000	125	>2000
	7	16 - 2000	125	>2000
	10	ND	ND	ND
	14	ND	ND	ND

MIC^{50} is an estimate of the concentration of antibiotic that inhibits 50% of the population of total *E.coli*. MIC^{90} is an estimate of the concentration of antibiotic that inhibits 90% of the population of total E.coli. ND – could not be determined from the counts on TBX supplemented with ciprofloxacin.

During the experiment in November 2012, within all microcosms before and after exposure to additional ciprofloxacin up until sampling day 7, *E.coli* could be detected on TBX supplemented with ciprofloxacin at levels between 16 and 2000 μg/L. This demonstrates that *E.coli* with and without ciprofloxacin acquired resistance (according to the epidemiological cut off value – non-wild type > 64 μg/L) in addition to resistant *E.coli* (according to clinical breakpoints - resistant > 1000 μg/L) were present in all microcosms over 7 days. *E.coli* could not be detected on TBX supplemented with ciprofloxacin at concentrations > 64 μg/L by day 10. However, the low number of total culturable *E.coli* found in the microcosms on sampling day 10 (see Figure 6-2) may have contributed to an apparent decrease in resistance by lowering the probability of detecting the resistant bacteria. Similar results were observed during the experiment commenced in July 2013, confirming that *E.coli* with acquired ciprofloxacin resistance mechanisms can survive in surface water microcosms for at least 7 days.

For both experiments, an increase in the estimated ciprofloxacin MIC_{50} value (from 16 to 125 μg/L) occurred following exposure to additional ciprofloxacin within microcosms exposed to 50 and 100 μg/L additional ciprofloxacin. No changes in the ciprofloxacin MIC_{50} occurred in all the other microcosms as shown in Tables 6-8 and 6-9. The estimated ciprofloxacin MIC_{90} values from all microcosms except those without exposure to additional ciprofloxacin, also demonstrated an increase following exposure to additional ciprofloxacin (from 125 to 2000 μg/L). Where detected, the elevated MIC_{50} and MIC_{90} values remained constant within microcosms until the counts of *E.coli* became too low to detect ciprofloxacin resistant *E.coli*.

6.3.4.4 The prevalence of ciprofloxacin resistance among E.coli within microcosms exposed to different levels of ciprofloxacin

The proportions (%) of total *E.coli* with acquired ciprofloxacin resistance measured in microcosms exposed to different levels of additional ciprofloxacin during the experiments commenced in November 2012 and in July 2013 are presented in Tables 6-10 and 6-11. Overall the trends observed from the microcosm experiments commenced in November 2012 were similar to those observed for the experiment carried out in July 2013.

Table 6-10: The proportion (%) of total *E.coli* with acquired ciprofloxacin resistance within microcosms exposed to different additional levels of ciprofloxacin over the course of the experiment in November 2012.

Exposure to additional ciprofloxacin (µg/L)	[a] % acquired resistance				
	[b] Day 0	Day 1	Day 7	Day 10	Day 14
0	12.1 ±4.4	15.0 ± 5.6	11.9 ± 0.9	ND	ND
5	9.6 ± 0.8	11.7 ± 2.7	20.5 ± 3.7	ND	ND
10	12.2 ± 20	18.6 ± 1.8	20.8 ± 3.7	ND	ND
50	12.1 ± 4.1	46.6 ± 12.7*	47.3 ± 5.2*	ND	ND
100	13.6 ± 0.1	54.4 ± 6.2*	51.7 ± 1.5*	ND	ND
[c] One-way ANOVA	$P = 0.657$	$P < 0.001$	$P < 0.001$	ND	ND

[a] average % ± standard deviation of triplicate microcosms. [b] Before exposure to additional ciprofloxacin. *Significantly different. [c] One way ANOVA with post hoc Tukey analysis. ND - could not be determined as *E.coli* counts on TBX supplemented with ciprofloxacin levels > 64 µg/L were below detection limits.

The proportion of total *E.coli* with acquired ciprofloxacin resistance was similar ($p > 0.05$) among all microcosms prior to exposure with additional ciprofloxacin. However, significantly elevated levels of acquired ciprofloxacin resistance ($p < 0.05$) occurred within microcosms

exposed to 50 and 100 μg/L additional ciprofloxacin compared to the other microcosms following exposure (see Tables 6-10 and 6-11).

Table 6-11: The proportion (%) of total E.coli with acquired ciprofloxacin resistance within microcosms exposed to different additional levels of ciprofloxacin over the course of the experiment in July 2013.

Exposure to additional ciprofloxacin (μg/L)	[a]% acquired resistance					
	[b] Day 0	Day 1	Day 3	Day 7	Day 10	Day 14
0	9.9 ±1.4	12.5 ± 3.0	15.5 ± 3.7	15.3 ± 6.1	ND	ND
5	10.7 ± 2.0	10.8 ± 0.7	30.4 ± 6.1	24.8 ± 5.0	ND	ND
10	10.5 ± 0.3	12.8 ± 11.3	29.2 ± 11.2	23.6 ± 8.6	ND	ND
50	9.7 ± 2.8	41.2 ± 13.5*	66.1 ± 5.6*	63.5 ± 9.8*	66.7 ± 1.8	ND
100	14.7 ± 0.5	60.9 ± 19.6*	75.5 ± 8.4*	50 ± 0.0*	ND	ND
[c] One-way ANOVA	$P = 0.09$	$P = 0.04$	$P < 0.001$	$P = 0.001$	ND	ND

[a] average % ± standard deviation of triplicate microcosms. [b] Before exposure to additional ciprofloxacin. *Significantly different. [c] One way ANOVA with post hoc Tukey analysis. ND - could not be determined as *E.coli* counts on TBX supplemented with ciprofloxacin levels > 64 μg/L were below detection limits.

This demonstrates a more pronounced effect of the influent antibiotic at concentrations close to the epidemiological cut off value (64 μg/L) on the prevalence of acquired resistance. The proportion of resistance within microcosms exposed to 50 and 100 μg/L additional ciprofloxacin remained high until the levels of *E.coli* became too low to evaluate the levels of acquired ciprofloxacin resistance (day 10).

6.3.4.5 The changes in proportion of total E.coli with acquired ciprofloxacin resistance with time

The proportion of *E.coli* with acquired ciprofloxacin resistance over the course of the experiments carried out in November 2012 and July 2013 are presented in Figure 6-3 A and

B. An overall comparison of the replicate experiments conducted in November 2012 and July 2013, reveals a trend in the prevalence of acquired ciprofloxacin resistance among microcosms. For both experiments the resistance levels, except for those without exposure to additional ciprofloxacin, demonstrated an increase. However, the level of acquired resistance remained constant ($P < 0.05$) within microcosms without additional ciprofloxacin exposure. In microcosms exposed to 50 and 100 μg/L additional ciprofloxacin, statistically elevated levels ($p < 0.05$) of acquired ciprofloxacin resistance were observed on sampling day 1 (after exposure). This was not surprising given that the total culturable *E.coli* population was significantly reduced within microcosms following exposure to 50 and 100 μg/L additional ciprofloxacin (as shown in Table 6-7).

Within all the constructed microcosms exposed to additional ciprofloxacin, the levels of acquired ciprofloxacin resistance peaked on sampling day 7 for the experiments carried out in November 2012 and day 3 for the experiments carried out in July 2013. Typically, the increase in the levels of resistance was found to be significant (one-way ANOVA with Tukey post hoc analysis – $p < 0.05$) as shown in Figure 6-3 A and B. Due to the low numbers of *E.coli* present within the microcosms on sampling days 10 and 14, the proportion of acquired ciprofloxacin resistance could not be measured and are therefore not presented in Figure 6-3.

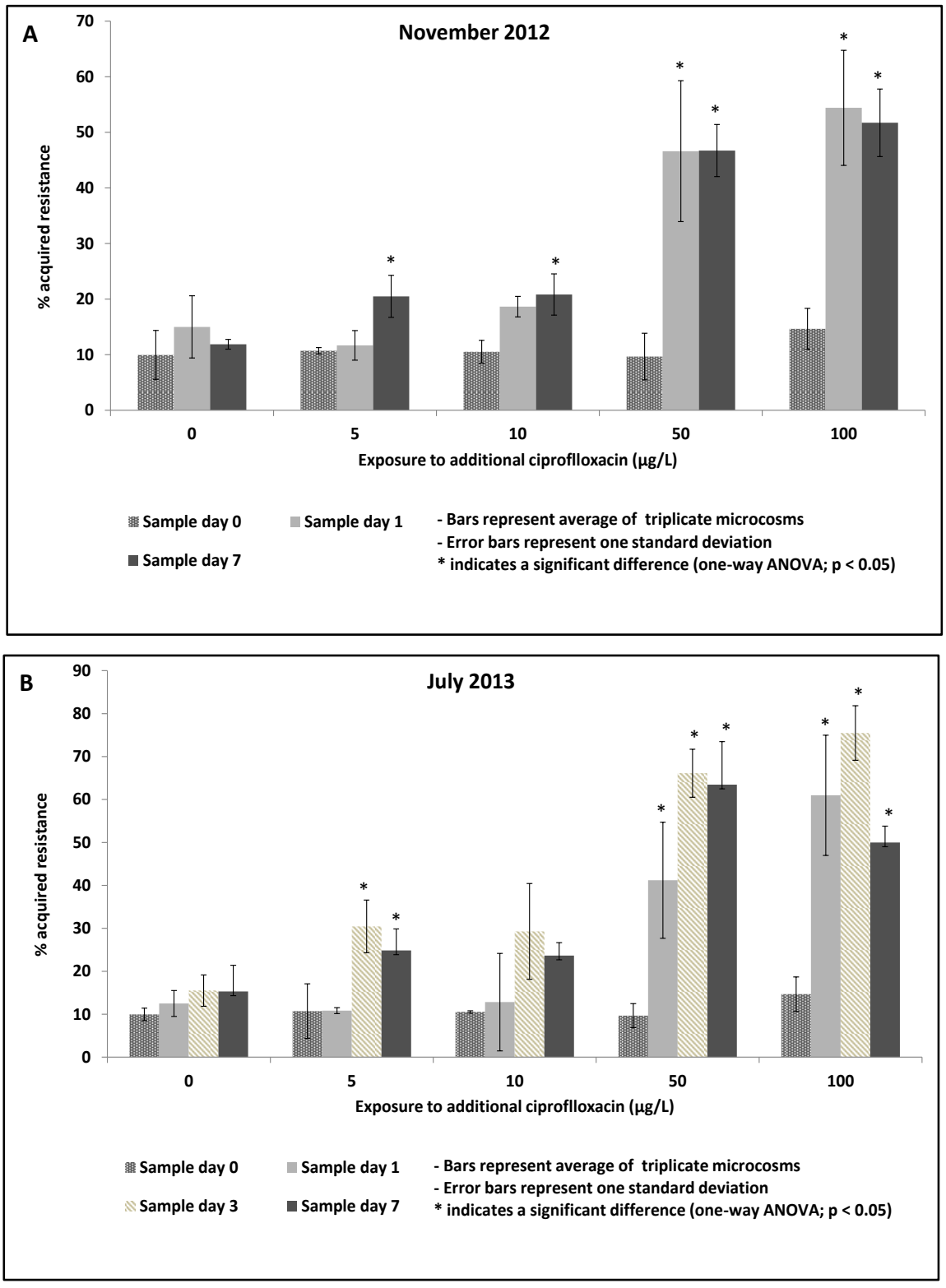


Figure 6-3: The proportion (%) of *E.coli* with acquired ciprofloxacin resistance within microcosms exposed to different levels of ciprofloxacin over time.

6.4 Discussion

6.4.1 Microcosm studies

There are a wide range of environmental variables (e.g. rainfall, background resistance, competitive bacteria and temperature) that may influence the prevalence of antibiotic resistance in surface waters making it a complex and challenging problem to monitor (Ashelford et al., 1997). Therefore, microcosm studies were used in this work as a simplified approach to study the prevalence of ciprofloxacin resistance among *E.coli* exposed to surface water receiving antibiotic residues within discharge treated wastewater effluent.

To exert the required experimental control over the microcosms, the experiments were carried out without light and at a constant dissolved oxygen concentration. In addition, the presence of protozoa were minimised within the constructed microcosms to delay the decline of total culturable *E.coli* over the course of the experiment (see Section 6.3.1) and thereby provide sufficiently high numbers of *E.coli* to enumerate through culturable techniques. However, it is important to take into account that predation by eukaryotic organisms may impact the level of *E.coli* resistance that occurs within natural waters (Bellanger et al., 2014).

6.4.2 Total culturable *E.coli*

At the beginning of the microcosm experiments, the concentrations of total culturable *E.coli* bacteria were similar in all microcosms. However, the concentration declined over the course of the experiment despite the use of protozoa inhibitor compounds. This was expected as *E.coli* bacteria are not indigenous to surface waters and therefore their survival

requires the ability to overcome an array of environmental stresses such as competitive bacteria and nutrient depletion (Pathak et al., 1994; Flint 1987).

The epidemiological cut off value defines an antibiotic inhibitory concentration value that distinguishes between wild type strains without acquired resistance mechanisms and non-wild type strains with acquired resistance (Kahlmeter et al., 2003). Exposure to antibiotic levels close to the epidemiological cut off value will eliminate wild type strains from the population. Therefore, it was expected that the impact of ciprofloxacin on the total culturable *E.coli* population would be more significant within microcosms exposed to ciprofloxacin levels close to the epidemiological cut off value (64 μg/L). The data from both microcosm experiments (commenced in November 2012 and July 2013) confirmed this hypothesis. The reduction in total culturable *E.coli* was significantly greater within microcosms exposed to 50 and 100 μg/L additional ciprofloxacin compared to microcosms exposed to lower levels of ciprofloxacin. This is similar to findings reported by Engemann et al. (2006) who observed that the size of the total bacterial community within simulated aquatic systems was significantly increased at greater levels of oxytetracycline (250 μg/L) compared to lower levels (25 μg/L).

During both experiments, *E.coli* could not be detected on sampling day 10 within microcosms exposed to 100 μg/L additional ciprofloxacin and this probably corresponds to the large reduction (~ 70.0 %) in *E.coli* observed following exposure to additional ciprofloxacin on sampling day 1.

6.4.3 Ciprofloxacin MIC determination

In both experiments, the range of ciprofloxacin minimum inhibitory concentration (MIC) values was the same for all microcosms until sampling day 10 when a smaller range of MIC values was observed. Although it appears there is a reduction of resistant *E.coli* on sampling day 10, the smaller MIC value range probably corresponds to the lower number of total *E.coli* and therefore a lower probability of detecting *E.coli* with higher ciprofloxacin MIC values. Despite the levels of ciprofloxacin, the range of MIC values were the same for all microcosms and spanned the concentrations that define wild and non-wild type *E.coli* and *E.coli* with MIC values that exceed the resistant breakpoint (1000 μg/L). This demonstrates resistant *E.coli* (according to both the epidemiological value and the clinical resistant breakpoint) are able to survive within the surface water microcosms irrespective of the level of ciprofloxacin exposure.

Increases in the estimated ciprofloxacin MIC_{90} (the concentration that can inhibit the growth of 90 % of the total *E.coli* population) values were observed within all microcosms exposed to additional ciprofloxacin indicating that the resistance can become more pronounced following the selective pressure of added ciprofloxacin levels of 5 μg/L or greater. Conversely, no change in the estimated ciprofloxacin MIC values was observed within microcosms without additional exposure. Similar trends were reported by Helt et al. (2011) who found the average ciprofloxacin minimum inhibitory concentration value for the culturable heterotrophic population within a simulated aquatic system increased (from 2000 – 4000 μg/L) following exposure to ciprofloxacin. Whereas, the MIC value of the heterotrophic

community in the simulated aquatic systems without ciprofloxacin exposure did not increase.

The apparent absence of a change in MIC values within microcosms without additional ciprofloxacin exposure confirms that the trace level antibiotic concentrations typically present in surface waters (ng/L) are sufficient to maintain the levels of ciprofloxacin resistance. It also indicates there may not be an associated fitness cost with the acquisition of ciprofloxacin resistance mechanisms even though the survival of *E.coli* is impaired in surface water due to competitive bacteria and decreasing nutrients.

6.4.4 Proportion of total *E.coli* with acquired ciprofloxacin resistance

Bacterial resistance to antibiotics is common where antibiotics are heavily used (e.g. clinical environments) (Andersson et al., 2012). However, it has been established that long-term exposure to low concentrations of antibiotics over time is likely to contribute to the selection of resistant bacteria (Andersson et al., 2014; Andersson et al., 2012; Gullberg et al., 2011). In addition, there are now increasing numbers of studies that report the presence of antibiotic resistant bacteria in wastewater contaminated surface waters, sediments and increasingly in surface water environments where the concentrations of antibiotics are present at sub-inhibitory concentrations (Faria et al., 2009; Pei et al., 2006; Ash et al., 2002; Guardabassi et al., 1998; Jones et al., 1986). Interestingly in this study, the introduction of ciprofloxacin at sub-inhibitory levels into surface water microcosms was found to have a considerable effect on the level of resistance (as presented in Figure 6-3).

The results from two experiments (commenced in November 2012 and July 2013) show the proportion of acquired ciprofloxacin resistance significantly increased within all microcosms

under the selective pressure of additional ciprofloxacin exposure concentrations at levels of 5 μg/L or above. This is concerning because although the antibiotic concentrations in surface water are typically lower than this exposure level, there are reports of ciprofloxacin concentrations as high as 9.6 μg/L in surface waters (Feitosa-Felizzola et al. 2009). The increase in the prevalence of acquired ciprofloxacin resistance could be due to two mechanisms. Either the exposure permitted the development of resistant strains though the dissemination of resistance elements or eliminated wild type strains allowing for the proliferation of non-wild type *E.coli* until resistance becomes dominant.

The increase in levels of *E.coli* with acquired ciprofloxacin resistance was more pronounced (one-way ANOVA – $p < 0.05$) with higher exposure concentrations (50 and 100 μg/L additional ciprofloxacin) and corresponds to a large reduction in the concentration of total culturable *E.coli* previously discussed in Section 6.4.2. The increased prevalence is therefore probably a consequence of non-wild type strains (with acquired ciprofloxacin resistance) out surviving their wild type (without acquired ciprofloxacin resistance) counterparts.

Simulated freshwater studies by Yu et al. (2009) found similar trends in the prevalence of resistant bacteria (*Enterococcus faecalis)* with exposure to antibiotics (ciprofloxacin and oxytetracycline were investigated). In addition, Helt et al. (2011) observed increases in the levels of ciprofloxacin resistant *E.coli* within constructed wetland mesocosm studies soon after exposure to 2 mg/L ciprofloxacin. However, the results from replicate mesocosms studies did vary. Contrasting levels of resistance within a simulated river water system due to an influent antibiotic were reported by Munoz-Aguayo et al. (2007). The levels of resistant bacteria did not increase following exposure to low concentrations (8 μg/L) of chlortetracycline. In addition, results from a study by Atoyan et al. (2007) demonstrated the proportion of

resistant bacteria within aerated and unaerated leachfield mesocosms did not increase upon the episodic exposure (every day for ten days) to 5 mg/L tetracycline. It was concluded that influent antibiotic concentrations are likely to have minimal consequences on *E.coli* bacteria within wastewater treatment processes. According to Helt et al. (2011) the contrasting results to this study may be a consequence of the different type of simulated system observed, the system conditions or a difference in the antibiotic resistance mechanisms being monitored. Additionally, the concentration of tetracycline used to define tetracycline resistance in *E.coli* by Atoyan et al. 2007 (10.0 mg/L) exceeds the epidemiological cut off value (8.0 mg/L) established by EUCAST (2012b). Therefore the level of resistance observed may have been underestimated. This provides another example of where a more consistent standardised approach to interpreting antibiotic resistance in environmental matrices is required. The interpretation of resistance surveillance has been discussed in more detail in Chapter 5, Section 5.4.2.

The proportion of total *E.coli* with acquired ciprofloxacin resistance within microcosms without additional ciprofloxacin exposure did not alter over the course of both experiments. This suggests that at ciprofloxacin concentrations typically detected in surface waters (ng/L), the dissemination of ciprofloxacin resistance elements does not occur within the *E.coli* population. This is not surprising given that studies such as those reported by Muela et al. (1994) found horizontal gene transfer processes within the *E.coli* population were inhibited in surface water conditions (e.g. low nutrient levels). However, it has been identified that the investigation of horizontal gene transfer processes within environmental matrices is challenging and there are a complex array of parameters that can affect plasmid transfer in the environment. These include the burden the transferred element may have on the

bacterial cell survival, predation, physiological conditions in addition to selective pressure (Bellanger et al., 2014).

Although the proportion of *E.coli* with acquired ciprofloxacin resistance did not increase within microcosms without additional ciprofloxacin exposure, the level was maintained. In addition to the ciprofloxacin MIC values discussed in Section 6.4.3 these findings suggest that *E.coli* bacteria with acquired ciprofloxacin resistance are not at any survival disadvantage compared to their wild type counterparts (strains without acquired resistance). Studies by (Caldwell et al., 1989; Flint, 1987) support this conclusion as they have shown resistance plasmids are stable within *E.coli* whilst under survival conditions in river water. In addition, studies by Enne et al. (2005) found that the burden of acquired resistance elements was small or non-existent to the survival of the certain strains of *E.coli* and suggested that once established, resistance may be difficult to eliminate through reduction in selective pressure. However, the fitness cost associated with acquired antibiotic resistance elements will depend on a number of factors such as the resistance element in question and the bacteria host and donor (Bellanger et al., 2014). It is also important to consider that genes encoding for antibiotic resistance are commonly found on the same plasmid as genes encoding for resistance to metals and disinfectants (Stepanauskas et al., 2006). In addition, plasmids containing multiple antibiotic genes have been identified (Szczepanowski et al., 2009) Therefore, the level of resistance may be maintained through coselection.

Although, an increase in the proportion of *E.coli* with acquired ciprofloxacin resistance was observed in microcosms following exposure to additional ciprofloxacin, the levels of

acquired ciprofloxacin resistance did not reach a maximum until sampling day three (during the experiment conducted in July 2013) and on sampling day seven (during the experiment conducted in November 2012). This is similar to the findings of Yu et al. (2009), who found maximum levels of antibiotic resistance within freshwater mesocosms three days after antibiotic (ciprofloxacin and oxytetracycline) exposure. The time lag in reaching the maximum proportion of acquired ciprofloxacin resistance could be due to the time required for the bacterial population to adapt to the microcosm source water ecology and ciprofloxacin concentration (Helt et al. 2011). In addition, plasmid mediated acquired ciprofloxacin resistance occurs through a step wise acquisition of QNr genes (Kaplan et al, 2013 and Cattoir et al, 2008) and the rate of this stepwise acquisition may account for the lag time observed. The observed rate at which the proportion of acquired ciprofloxacin resistance reached a maximum obviously differed between the experiments conducted in November 2012 and July 2013 due to the fewer sampling dates carried out in November 2012.

In the present study the prevalence of acquired resistance could not be interpreted beyond sampling day 7. However, Yu et al. (2009) and Helt et al. (2011) both found that following a single exposure to an influent antibiotic, resistance levels within a bacterial population eventually decrease over time, indicating antibiotic resistance can be eliminated. In addition, Sorensen et al. (2005) reported that resistant traits can decline within a bacterial population with time when the selective pressure is removed. However, this maybe a gradual process if the traits do not pose a metabolic burden to the bacterial strain. Further studies investigating the episodic release of antibiotic residues within treated wastewater

effluent discharges to receiving waters on the maintenance of resistance within a bacterial population would need to be performed to investigate this further.

Overall, the results from this work demonstrate that discharges of antibiotic residues have the potential to maintain or even increase the prevalence of acquired resistance in surface waters. This is of great concern especially for countries such as China and India where substantial quantities of antibiotics are manufactured and the reported concentrations of antibiotics (e.g. ciprofloxacin) in environmental waters can be a 1000 fold greater than those typically reported for Europe (Diwan et al., 2010 and Wei et al., 2012). In one monitoring study in India, ciprofloxacin concentrations as high as 2,500 μg/L were reported in river water down-stream of a waste water treatment plant which are greatly elevated compared to those used in this microcosm study (5 – 100 μg/L) (Soderstorm et al, 2009). It is therefore clearly evident that the maintenance of antibiotic resistance and the potential to disseminate resistance should be considered in environmental risk assessments as this is ultimately a concern to public health worldwide. The efficiencies of wastewater treatment processes with regard to the removal of antibiotic residues need to be improved to contribute to the reduction in the potential of resistant bacteria to proliferate in surface waters that receive wastewater treated effluent discharges.

6.5 Summary

The results from two individual microcosm experiments simulating surface water receiving treated wastewater effluent indicate the potential of sub-inhibitory ciprofloxacin levels to influence the prevalence of acquired ciprofloxacin resistance among *E.coli*. This study supports the findings by Helt et al. (2011) and Yu et al. (2011) which suggest that

ciprofloxacin can promote the prevalence of antibiotic resistant *E.coli* within surface waters. The results demonstrate that at ciprofloxacin levels typically detected in surface waters the prevalence of acquired ciprofloxacin resistance among *E.coli* does not increase. However, the prevalence of resistance is maintained. Thereby indicating non-wild type strains are not at a survival disadvantage compared to their wild type counterparts.

7 Conclusion

7.1 Occurrence of pharmaceuticals in environmental waters

The presence of pharmaceutical compounds in the environment has become a global concern over recent years, due to their ubiquitous presence and their pseudo-persistency. It is imperative that we understand the fate and effects of these compounds in the environment, in order to lessen their impact.

In this study, the analysis of prescription data has indicated the high quantities of four pharmaceuticals (bezafibrate, carbamazepine, ciprofloxacin and clarithromycin) prescribed per year that could ultimately arrive at wastewater treatment plants following ingestion and excretion. The analysis of sewage and final effluents confirmed these compounds are present within wastewater and demonstrated that wastewater treatment processes do not completely eliminate these compounds. Although the percentage pharmaceutical removal during wastewater treatment depends on a number of factors including the type of wastewater treatment and the catchment population characteristics, sorption during activated sludge treatment is shown to be an important removal process for ciprofloxacin. The treatment and disposal of activated sludge material in addition to the discharges of treated effluent to surface waters must therefore be considered as part of thorough environmental risk assessments.

A comparison of pharmaceutical concentrations in surface waters up- and down-stream of the final effluent discharge point from a wastewater treatment plant confirm these compounds are not completely removed during wastewater treatment. Therefore surface

waters are vulnerable to pharmaceutical contamination from point sources. This is a concern as the monitoring of these compounds in natural waters is not enforced.

Current legislation pertaining to pharmaceutical compounds in the environment requires that an environmental risk assessment is conducted on all new medicinal products before market approval. However, the pharmaceutical compounds currently on the market are exempt. Therefore the information on the fate and effects of these pharmaceuticals (including bezafibrate, carbamazepine, clarithromycin and ciprofloxacin) is incomplete. This is of concern as there are reports indicating the potential risk of these selected compounds to aquatic ecosystems. In addition, there is currently no recommendation for tailored tests to investigate the effects that may be specific to certain groups of pharmaceuticals. The effects of antibiotics in aquatic ecosystems and the potential to select for antibiotic resistant bacteria is an example.

7.2 Antibiotic resistance profiles of faecal indicators in environmental waters

In this work, *E.coli* and *E.faecium* were isolated from settled sewage, treated effluent and receiving surface waters to investigate the prevalence of antibiotic resistance amongst these bacteria. *E.coli* was easily selected and differentiated from other enterobacteriaceae using β- galactosidase/β glucuronidase chromogenic media. The use of Slanetz and Bartley media for the detection and enumeration of enterococci can facilitate the growth of other bacteria physiologically similar to enterococci and therefore does not enable the distinction between different enterococci species. However, with additional identification using MALDI-TOF-MS

analysis it was demonstrated that *E.faecium* were present in high enough proportions in the wastewater and surface water to study antibiotic resistance profiles.

The comparison of the MIC values determined for *E.coli* with both harmonised clinical breakpoint and epidemiological cut off values demonstrated there is an urgent need for the standardisation of the interpretive criteria used for assessing antibiotic susceptibility data. Different conclusions could have been reported if the prevalence of resistant *E.coli* in wastewater and surface waters were interpreted using clinical breakpoints. The standardisation and harmonisation of values used to define resistance are imperative for effective surveillance of antibiotic resistance development. A single standardised approach is crucial for effective risk assessment and a comparison of the derived resistance from different sources (e.g. food, environment and humans) is required. The use of quantitative minimum inhibitory concentration data and interpretation using epidemiological cut off values enables the distinction of subtle changes in resistance and is therefore recommended. In addition, quantitative data is perhaps more useful for researchers to compare their findings.

Despite the large percentage of faecal indicators that were removed during the wastewater treatment process, it was found that wastewater treatment did not significantly change the proportion of *E.coli* or *E.faecium* resistance to antibiotics. This indicates the level of resistance is maintained throughout wastewater treatment processes but does not provide evidence that wastewater treatment plants are hotspots for the dissemination of resistance. However, it was demonstrated that the discharges of treated wastewater effluent influenced the levels of antibiotic resistant bacteria in receiving surface water. The

increased prevalence of resistant bacteria suggests either the exposure to antibiotic residues within treated effluent permitted the development of resistant strains through the dissemination of resistance elements, or through the elimination of wild type strains, allowing the proliferation of non-wild type bacteria until resistance becomes dominant.

7.3 Ciprofloxacin resistant profiles of *E.coli* in surface water microcosms

The effects of antibiotics in aquatic ecosystems and the potential to select for antibiotic resistant bacteria were investigated through microcosm studies. The results obtained for the level of antibiotic resistance among *E.coli* isolates within surface water microcosms supports the idea that the introduction of an antibiotic to an aquatic system can lead to an increase in resistance. A significant increase in the level of ciprofloxacin resistance amongst *E.coli* was observed in microcosms exposed to antibiotic levels of 5 μg/L or greater during two replicate experiments. This is of concern given that antibiotic residue concentrations as high as this have been detected in surface waters receiving wastewater treated effluent discharges.

Interestingly, at antibiotic residue levels typically detected in surface waters receiving treated wastewater effluent (~ < 5 ng/L - 200 ng/L), the levels of resistance amongst *E.coli* did not significantly alter. This demonstrates that the survival of non-wild type *E.coli* with acquired resistance mechanisms is not impaired compared to wild type *E.coli*. The findings presented in this work highlight that wastewater treatment processes to remove antibiotic residues need to be developed. This will reduce the potential of the proliferation of resistant bacteria in surface waters that receive wastewater treated effluent discharges.

7.4 Thesis recommendations and future work

This research clearly shows that pharmaceutical compounds (including antibiotics) are not efficiently removed during wastewater treatment processes and consequently elevated concentrations are found in receiving surface waters. However, it is still unclear what the full impact of these compounds maybe on aquatic ecosystems. The assessment of the environmental risk that these compounds may pose for aquatic ecosystems should be more comprehensive and include tests that target specific effects such as the dissemination of antibiotic resistance. Furthermore, additional wastewater treatment processes should be developed and introduced if environmental risk assessments do indicate complete removal of these residues is required.

The findings presented in this work demonstrate wastewater treatment processes maintain the levels of antibiotic resistance among faecal indicators. Additionally, results indicate that the discharge of treated effluents affect the levels of resistant faecal indicators within surface waters receiving treated effluent discharges. The dissemination of antibiotic resistance is of great concern and will impact both human and animal medicine as the threat to antibiotic therapy increases. Therefore the surveillance of antibiotic resistance in the environment, in addition to the surveillance already carried out in clinical environments, is imperative. In addition, epidemiological studies within wastewater treatment plants employing advanced treatment options should be considered. Bacteria in addition to those typically found in human gut flora should also be studied. A focus on bacteria indigenous to our surface waters such as Aeromonas species should be considered. However, it is necessary to standardise the interpretation of resistance in the environment and to consider interpretative criteria such as epidemiological cut off values to avoid underestimating subtle changes in antibiotic resistance.

In addition, a closer look into the cause of the increase of antibiotic resistance proportions would provide useful data. Is it the antibiotic residues or the excretion of antibiotic resistant bacteria the cause of elevated resistance proportions in surface waters down-stream from wastewater treated effluent discharge points.

Simple microcosm studies have demonstrated that antibiotic concentrations at levels typically present in surface waters receiving discharges of wastewater treated effluents are sufficient to maintain the proportion of antibiotic resistance among faecal bacteria. In addition, it has been demonstrated that sub-inhibitory levels of antibiotics can significantly increase the prevalence of resistance. Additional microcosm studies to investigate the selective effects of antibiotic residues on the prevalence of resistance among a variety of waterborne bacteria will be useful to gain a more comprehensive representation of surface waters. It would also be an advantage to look at bacteria that are indigenous to surface waters in addition to faeceal bacteria. Microcosm studies that investigate the proportion of resistant bacteria in source water without antibiotic contamination would be useful as would an investigation into the spread of co-resistance through the presence of other pollutants such as metal contamination. In addition, studies that look at the effects of protozoa and UV light on the spread of resistance would provide more information. Furthermore, a variety of antibiotics and their influence on the prevalence of multiple resistances needs to be considered. In addition, molecular studies although challenging would be useful to investigate the mechanisms of the dissemination of resistance in surface waters and to establish how stable and transferable resistance elements are once they have entered surface waters. For example, antibiotic resistance gene abundances could be monitored in a similar microcosm study set ups and the rate of plasmid mediated resistance acquisition could also be studied. Investigating antibiotic resistance in bacteria that cannot be cultured through techniques such as functional metagenomics would be invaluable to

this area of work. The use of simple microcosm's studies could be improved to include biofilms, and enable the investigation of antibiotic resistance in water circulating microcosm studies to better simulate the river flow conditions and the water/ sediment mixing conditions found in surface waters.

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Appendix 1

Biochemical tests included in the API 20E system (Biomerieux, 2005)

Test	Media substrate	To detect the following Enzyme activity/ reaction	Positive reaction	Negative reaction
ONPG	2-nitrophenyl-ßD-galactopyranoside	ß-galactosidase	Yellow	No colour
ADH	L-arginine	Arginine Dihydrolase	Yellow	red
LDC	L-Lysine	Lysine decarboxylase	Yellow	red
ODC	L-ornithine	Ornithine Decarboxylase	Yellow	red
CIT	trisodium citrate	citritase	Pale yellow	Blue
H_2S	sodium thiosulfate	Production hydrogen sulfide	Colourless	Black
URE	Urea	Urease	Yellow	Red
TDA	L-Tryptophan	Tryptophane DeAminase	Yellow	Brown
IND	L-Tryptophan	Tryptophane DeAminase	Colourless	Pink
VP	sodium pyruvate	Acetyl-methyl-carbinol production	Colourless	Pink
GEL	Gelatin (bovine origin)	Gelatinase	No diffusion	Diffusion
GLU	D-Glucose	Fermentation-oxidation	Blue	Yellow
MAN	D-Mannitol	Fermentation-oxidation	Blue	Yellow
INO	D-Insitol	Fermentation-oxidation	Blue	Yellow
SOR	D-Sorbitol	Fermentation-oxidation	Blue	Yellow
RHA	L-Rhamnose	Fermentation-oxidation	Blue	Yellow
SAC	D-Saccharose	Fermentation-oxidation	Blue	Yellow
MEL	D-Melibose	Fermentation-oxidation	Blue	Yellow
AMY	Amydaline	Fermentation-oxidation	Blue	Yellow
ARA	L-Arabinose	Fermentation-oxidation	Blue	Yellow

The oxidase test is included in the profile

Appendix 2

Biochemical tests included in the API 20 Strep system

Test	Media substrate	To detect the following Enzyme activity/ reaction	Positive reaction	Negative reaction
VP	sodium pyruvate	Acetyl-methyl-carbinol production	Colourless	Pink
HIP	Hippuric acid	Hydrolysis by Hippuricase	Colourless	Dark blue
ESC	Esculin ferric citrate	Hydrolysis of esculin by B-Glucosidase	Colourless	Black
PYRA	Pyroglutamic acid β-naphthylamide	Pyrorolidonyl arylamidase activity	Colourless	Orange
αGAL	6-bromo – 2 – naphthyl-αD-galactopyranoside	α Galactosidase activity	Colourless	Violet
βGUR	Naphthol ASBI-glucuronic acid	β Glucuronidase activity	Colourless	Blue
βGAL	2-naphthyl - β Galactopyranoside	β Galactosidase activity	Colourless	Violet
PAL	2-naphthyl phosphate	Alkaline Phosphatase	Colourless	Violet
LAP	L-leucine-ß-naphtylamide	Leucine AminoPeptidase	Colourless	orange
ADH	L-arginine	Arginine Dihydrolase	Yellow	red
RIB	D-ribose	Acidification	Red	Yellow
ARA	L-Arabinose	Fermentation-oxidation	Blue	Yellow
MAN	D-Mannitol	Fermentation-oxidation	Blue	Yellow
LAC	D-lactose	Acidification	Red	Yellow
TRE	D-trehalose	Acidification	Red	Yellow
INU	Inulin	Acidification	Red	Yellow
RAF	D-Raffinose	Acidification	Red	Yellow
AMD	Starch	Acidification	Red	Yellow

Appendix 3

Biochemical tests included in the API 20 Staph system

Test	Media substrate	To detect the following Enzyme activity/ reaction	Positive reaction	Negative reaction
0	No substrate	Negative control	Red	-
GLU	D-glucose	Positive control	Red	Yellow
FRU	D-fructose	Acidification	Red	Yellow
MNE	D-mannose	Acidification	Red	Yellow
MAL	D-maltose	Acidification	Red	Yellow
LAC	D-lactose	Acidification	Red	Yellow
TRE	D-trehalose	Acidification	Red	Yellow
MAN	D-mannitol	Acidification	Red	Yellow
XLT	Xylitol	Acidification	Red	Yellow
MEL	D-melibiose	Acidification	Red	Yellow
NIT	potassium nitrate	Reduction of nitrates to nitrites	Colourless	Red
PAL	ß-naphthyl phosphate	alkaline Phosphatase	Yellow	Violet
VP	sodium pyruvate	Acetyl-methyl-carbinol production	Colourless	Pink
RAF	D-raffinose	Acidification	Red	Yellow
XYL	D-xylose	Acidification	Red	Yellow
SAC	D-saccharose	Acidification	Red	Yellow
MDG	methyl-αDglucopyranoside	Acidification	Red	Yellow
NAG	N-acetyl-glucosamine	Acidification	Red	Yellow
ADH	L-arginine	Arginine dihydrolase	Yellow	Red
URE	urea	urease	Yellow	Red

The acidification tests should be compared to the negative (0) and positive (GLU) controls.

Appendix 4

Biochemical tests included in the API 20NE system

Test	Media substrate	To detect the following Enzyme activity/ reaction	Positive reaction	Negative reaction
NO_3	Potassium nitrate	Reduction nitrate to nitrites	Colourless	Pink
GLU	D-Glucose	Fermentation-oxidation	Pink	Colourless
TRP	L-tryptophane	Indole production	Colourless	Pink
ADH	L-arginine	Arginine Dihydrolase	Blue	Yellow
URE	urea	urease	Yellow	Orange/red
ESC	Esculin ferric citrate	Hydrolysis of esculin by B-Glucosidase	Yellow	Orange/red
PNPG	4-nitrophenyl-β D-galactopyranoside	β-galactosidase (Para-NitroPhenyl-ßD-Galactopyranosidase)	Yellow	Brown/black
GEL	Gelatin (bovine origin)	Gelatinase	No diffusion	Diffusion
GLU	D-Glucose	Assimilation	Transparent	opaque
ARA	L-Arabinose	Assimilation	Transparent	opaque
MNE	D-mannose	Assimilation	Transparent	opaque
MAN	D-mannitol	Assimilation	Transparent	opaque
NAG	N-acetyl-glucosamine	Assimilation	Transparent	opaque
MAL	D-maltose	Assimilation	Transparent	opaque
CAP	Capric acid	Assimilation	Transparent	opaque
ADI	adipic acid	Assimilation	Transparent	opaque
MLT	Malic acid	Assimilation	Transparent	opaque
CIT	trisodium citrate	Assimilation	Transparent	opaque
PAC	phenylacetic acid	Assimilation	Transparent	opaque

The oxidase test is included in the profile

Appendix 5

Presentations

1. Rebecca Tuckwell, Mike Revitt, Huw Jones and Hemda Garelick, Distribution of minimum inhibitory concentrations to antibiotics in *E.coli* and Enterococci isolated from wastewater effluents and surface waters, Middlesex Summer Comference, The School of Health and Social Sciences (HSSc), Middlesex University, London, June 2012.
2. Rebecca Tuckwell, Mike Revitt, Huw Jones and Hemda Garelick, Sources and pathways for pharmaceuticals in the urban water environment, 12th International Conference on Urban Drainage, Porto Alegre/Brazil, 11-16 September 2011.
3. Rebecca Tuckwell, Mike Revitt, Huw Jones and Hemda Garelick, Sources and pathways for pharmaceuticals in the urban water environment, Middlesex Summer Conference, The School of Health and Social Sciences (HSSc), Middlesex University, London, June 2011.

Poster presentations

1. Rebecca Tuckwell, Mike Revitt, Huw Jones and Hemda Garelick, Distribution of minimum inhibitory concentrations to antibiotics in E.coli and Enterococci isolated from wastewater effluents and surface waters, 6th SETAC World Congress / SETAC Europe 22nd Annual Meeting, Berlin, 20th - 24th May 2012.